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Studies on the Essential Oils of Tobacco Leaves

Part IV. Neutral Fraction

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Received January 17, 1956

An investigation was conducted on the neutral fraction in the essential oil of Virginia tobacco leaves. α-Pyrryl methyl ketone, ethyl alcohol, ethyl acetate and furfuryl alcohol were isolated and identified. An alcohol, C_4, and an ester of benzoic acid were also separated. Hydrocarbons were separated from the total neutral fraction by liquid chromatography, prior to any further procedure; and a straight chain paraffin near C_{30} and an unsaturated hydrocarbon resembling myrcene were also isolated and their contents were determined. A technique of chromatostrip was found to be very useful for detecting liquid chromatographic separation.

Among the neutral compounds contained in tobacco essential oils, the following compounds have been reported: as for alcohols found partly free and partly esterified, C_4 and C_6 aliphatic alcohols[1], terpene alcohols[2], furfuryl alcohol[2,3] and polyhydric alcohols such as glycerol[4]. As for hydrocarbons, $C_{10}H_{16}$[2], $C_{10}H_{18}$ and $C_{11}H_{20}$[5] were estimated, these probably representatives of the class of terpenes, and paraffins $C_{27}H_{56}$ and $C_{31}H_{64}$[3]. This paper deals with the isolation and identification of individual compounds contained in the neutral fraction of essential oil in fermented Virginia tobacco leaves.

More than sixty percent of the essential oil of Virginia tobacco leaves consisted of the neutral fraction[6]. This fraction manifests the fundamental odor of fermented Virginia tobacco leaves.

By means of two dimentional chromatostrip technique[7,8], using 15% ethyl acetate in *n*-hexane and chloroform as each dimentional developing solvent, the neutral fraction, obtained by the procedure in the previous paper[6], was found to contain at least nine components. The characteristic appearance on the chromatograms proved that not any predominant components were present in this neutral fraction. Low boiling point fractions (α)[9], under 100°, were removed beforehand by distillation with an acetone-dryice acceptor so as to avoid the loss in the separation of the chromatographic eluting solvent. In order to separate free alcohols from this distillate, 3,5-dinitrobenzoylchloride was reacted directly, and the products were chromatographed by the method of White and Dryden[10]. Thus ethyl alcohol was identified and C_4 alcohol was

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1) A. Shmuk, *Inst. Exptl, Tobacco Culture, Krasnodar*, **19**, 1 (1924): *C. A.*, **20**, 967 (1926).

2) W. G. Frankenburg, *Advances in Enzymology*, **6**, 331 (1946).

3) Y. Fukushima, *J. Chem. Soc, Japan*, **61**, 1297 (1940); **62**, 413 (1941).

4) O. Lazar and A. H. Meyling, *J. S. African Chem. Inst.*, **21**, 8 (1938); *C. A.* **32**, 8688 (1938).

5) W. Halle and E. Pribram, *Ber.*, **47**, 1294 (1914).

6) I. Ōnishi and K. Yamasaki, This Bulletin, **19**, 139 (1955).

7) J. G. Kirchner, J. M. Miller and G. J. Keller, *Anal. Chem.*, **23**, 420 (1951).

8) This method was modified as follows: silicic acid (10g., Mallincrodt Analytical Reagent, No. 2847, 100 mesh, specially prepared for chromatography), mixed with potato starch (0.6g., Japan Pharmacopoeia) with 20 ml. of distilled water until the starch had coagulated and the mixture was favored a thick paste.

9) These notes correspond to the names of fractions indicated in Table III.

10) J. W. White and E. C. Dryden, *Anal. Chem.*, **20**, 853 (1948).

isolated. The unreacted part with the reagent was saponified and ethyl alcohol and acetic acid were identified, thus the presence of ethyl acetate was confirmed. After separation of the low boiling fraction, the hydrocarbons were separated from the oxygenated compounds by liquid chromatography, employing silicic acid as the adsorbent and *n*-hexane as the eluting solvent[7,11,12,13]. The border, whether the hydrocarbons were eluted out or not, was detected by a color reaction and infrared spectrum of the eluates. The hydrocarbons were divided into paraffins (β) and unsaturated ones (γ) by application of their difference of solubility in ethyl alcohol, further separation being conducted by liquid chromatography. From the paraffin fraction, triacontane (or hentriacontane) was isolated. The unsaturated hydrocarbons consisted of four individual components, and the infrared spectrum[14] of the predominant component indicated that this unsaturated hydrocarbon has a very close resemblance to myrcene. And then, about eleven percent of the neutral fraction consisted of hydrocarbons of which at least four or more percent of the hydrocarbons are saturated.

TABLE I
FRACTIONAL DISTILLATION OF A HIGH BOILING POINT FRACTION OF OXYGENATED COMPOUNDS IN THE NEUTRAL FRACTION

Fr.	b.p. (°C)	pr. (mm.Hg.)	bath temp. (°C)	yield (g.)
1	45– 75	3.5	180–191	0.0953
2	79– 98	〃	191–203	0.5728
3	98–100	〃	203	0.8391
4	100–114	〃	203–215	0.5370
5	114–132	〃	215–236	0.3200
6	132–143	〃	236–245	3.4953
7	143–150	〃	245–265	0.4182

The oxygenated compounds were distilled fractionally, the results are shown in Table I. High boiling fractions of the oxygenated compounds, a dark reddish viscous oil, had a sweet and aromatic odor of original tobacco essential oil. The physical and chemical constants were as follows: d^{20}_{20} 0.9943, n^{20}_{D} 1.5061 and the saponification value 64.7. It was found clear that fractionation by distillation when detected by the method of chromatostrip was not so completely performed. So we collected the fractions into two lumping groups, having characteristic and common spots on the chromatostrips such as Fractions 1 to 5 or Fractions 6 to 7, which are indicated in Table I. Each of the groups was separated by liquid chromatography, using a mixture of silicic acid and celite[15] (2 : 1, by weight) as the adsorbent, with a mixture of *n*-hexane and ether as the eluting solvent. The results are shown in Table II. Removal of the solvent from the eluate of $(\mu)_9$ yielded long needle crystals. After recrystallization from *n*-hexane, a fine white needle crystal was obtained. The mixed melting point, elementary analysis and infrared spectrum confirmed that this needle crystal is α-pyrryl methyl ketone. Furthermore, the melting

TABLE II
CHROMATOGRAPHIC SEPARATION OF FRACTIONAL DISTILLATES OF OXYGENATED COMPOUNDS IN THE NEUTRAL FRACTION

	(μ) fraction (F_1-F_5) (45–132°/3.5 mm.)	(p) fraction (F_6-F_7) (132–150°/3.5 mm)
1	214.0 mg.	422.8 mg.
2	164.6	483.6
3	35.7	339.2
4	200.8	293.6
5	126.3	98.9
6	32.4	251.2
7	76.9	286.9
8	111.2	309.6
9	78.3	256.0
10	195.7	483.0
11	54.4	80.2
12	60.9	260.3

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[11] J. G. Kirchner and J. M. Miller, *Ind. Eng. Chem.*, **44**, 318 (1952).

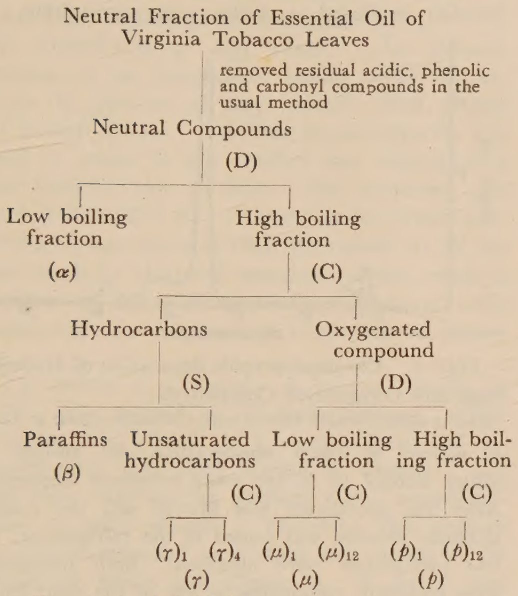
[12] J. M. Miller and J. G. Kirchner, *Anal. Chem.*, **24**, 1480 (1952).

[13] J. M. Miller and J. G. Kirchner. *Anal. Chem.*, **25**, 1107 (1953).

[14] All the infrared spectra were obtained with a Perkin-Elmer Model 21 infrared spectrophotometer installed in our research institute.

[15] Johns-Manville

TABLE III
FRACTIONATION OF THE NEUTRAL FRACTION



(D) : fractional distillation
(C) : chromatographic separation
(S) : separation by solubility

point and the infrared spectrum of its 2,4-dinitrophenylhydrazone were identical with that of the authentic α-pyrryl methyl ketone. On the other hand, benzoic acid was isolated and identified from another eluate, after saponification. These separating procedures are schematically shown in Table III.

In order to refine the chromatographic eluting solvent, a packed column, described in the previous paper[6], was used. All the distillations were undertaken with a Claisen flask in a nitrogen stream, so that the deterioration might be minimized during the distillations. Hence, all the liquid chromatographic procedures were operated under compressed nitrogen pressure, using the apparatus illustrated in the previous paper[16]. The chromatostrip method, reported by Kirchner and Miller, proved to be very useful, because the development completed within half an hour, the separation of spots also being generally clear. Furthermore, the characteristic coloration is very effectual for estimating the components, by using a mixture of concentrated sulfuric acid and nitric acid (19 : 1, v/v) as a coloring reagent. For the detection of 3,5-dinitrobenzoates of alcohols on chromatostrips, α-naphthylamine was used as a coloring reagent[17].

Without these procedures, such as distillation or chromatography, the original neutral compounds were saponified directly, and furfuryl alcohol was isolated and identified from the alcoholic fraction.

It has generally been considered that any compounds containing nitrogen have a strong activity making tobacco smoke pungent and irritating. Nevertheless, α-pyrryl methyl ketone has no such disagreeable character, even though it has a rather high content of nitrogen. Furthermore, on the contrary, it is a particularly noteworthy fact that α-pyrryl methyl ketone makes tobacco smoke mild, whether it is used for cut tobacco of cigarette in a proper small quantity or a large amount, about 0.1 or over one percent, and it can be smoked without any disagreeable remarks. This specific phenomenon seems to be mainly caused by its chemical character having a neutral reaction and easy volatility.

Furthermore, it has a somewhat faint aromatic odor, resembling cinnamone, followed by diluted almond oil bitter: it was also reported that it has a sweet and in no way any disagreeable pungent taste[18]. When added to cut tobacco for cigarettes in a moderate proportion, the aromatic odor turns stronger than that of the original crystalline form.

On the other hand, it has a strong narcotic activity[19,20], but this is less toxic[21]. The fatal dose of nicotine is reported to be as less than one milligram per one kilogram of horse body weight[22], but that of α-pyrryl methyl ketone is eight hundred milligrams for mice[21].

---

[16] I. Ōnishi and K. Yamamoto, This Bulletin **19**, 149 (1955).

[17] R. G. Rice. G. J. Keller and J. G. Kirchner, *Anal. Chem.*, **23**, 194 (1951).

[18] C. Riccomanni, *Atti accad. Lincei*, **33**, 145 (1924); *C. A.*, **18**, 2900 (1924).

[19] A. Rabbeno, *Arch. intern. pharmacodynamie* **35**, 377; *Chem. Zentr.* 1929, **II**, 1318; *C. A.*, **25**, 145 (1931).

[20] A. Rabbeno, G. Rastelli and S. Sacchi, *Boll. soc. ital. biol. sper.*, **13**, 167 (1938); *C. A.*, **32**, 5070 (1938).

[21] J. V. Supniewski and E. Taschner, *Bull. intern. acad. polon sci. classe med.*, 1935, 627; *Ber. ges. Physiol exptl. Pharmakol.*, **95**, 380; *C. A.*, **32**, 9279 (1938).

Recently, mild and low nicotine tobaccos are exceedingly widely spread throughout the world, in fear of the toxicity of nicotine. However, low nicotine tobaccos cause a very ineffective feeling on smoking. In such cases, α-pyrryl methyl ketone would make the tobacco smoke mild and effective, because of its chemical characters and strong narcotic activity.

Anyhow, it is an interesting fact that such a specific compound as α-pyrryl methyl ketone is to be found in tobacco essential oil.

EXPERIMENTAL

(I) Hydrocarbons

(1) Separation of hydrocarbons: In order to separate hydrocarbons from oxygenated compounds, the chromatographic method, according to Kirchner and Miller[11], was employed. Silicic acid, 6.0 g., heated at 210° for 6 hours before use, was packed into a small column as a slurry in *n*-hexane, b.p. 67–68°, which was purified by a rectifying column as described in the previous paper[6]. Neutral compounds, 206 mg., was added and developed using *n*-hexane as the eluting solvent. The effluents were collected at an interval of 0.5 ml. The border was determined by a color reaction with concentrated sulfuric acid whether or not the hydrocarbons eluted out perfectly. After the reaction was found to be negative, the eluting solvent was changed to ethyl acetate[23] which was purified by the same rectifying column as used for *n*-hexane. The solvent was removed at 0° by evaporation at reduced pressure, 10 mm. Hg. Each residue was weighed one by one. Figure 1. shows the separation of the hydrocarbons and oxygenated compounds.

(2) Isolation of triacontane or hentriacontane: The hydrocarbon fraction was dried over anhydrous sodium sulfate and evaporated off the solvent. The yield of this fraction indicates that hydrocarbon comprises ca. 11% of the total neutral compounds. On standing in the refrigerator, the hydrocarbon fraction produced a white waxy precipitate, but it melted at room temperature. By adding absolute alcohol to it, the waxy substance reappeared. After the precipitate was filtered off, the residual alcoholic solution was cooled in the refrigerator, and few precipitates were obtained. Both precipitates were collected, comprising as 4% of the total hydrocarbons, then after repeated recrystallization from acetone and benzene, a white flaky crystal, m.p. 65.0–66.2°[24], was obtained. An infrared spectrum of this crystal is identical with that of straight chain higher paraffin. The melting point and elementary analysis indicated that it might be triacontane, m.p. 66°, or hentriacontane, m.p. 68°. *Anal.* Found: C, 85.25; H, 15.01. Calcd. for $C_{30}H_{62}$: C, 85.22; H, 14.78 and for $C_{31}H_{64}$: C, 85.23; H, 14.77.

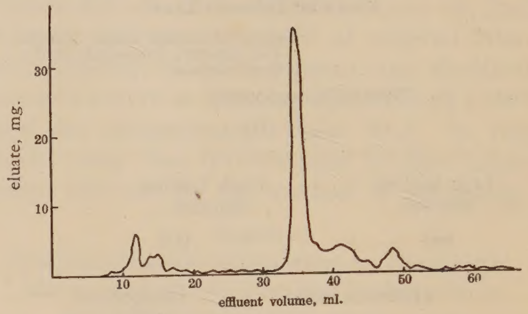


FIG. 1. Chromatographic Separation of Hydrocarbons and Oxygenated Compounds.

Eluting agent changed from *n*-hexane to ethyl acetate at 25 ml.

(3) Estimation of myrcene like unsaturated hydrocarbon: In order to separate residual paraffins, the fraction soluble in alcohol was distilled. The distillate was separated chromatographically into four fractions using silicic acid as the adsorbent and *n*-hexane as the developer. The first eluate fraction was the main constituent of the distillates and comprised 60.2% of the unsaturated hydrocarbons. M.W. 177.91, d_{15}^{15} 0.8204, n_D^{15} 1.4690, M.R. 60.3932. Its infrared spectrum shows a close resemblance to that of myrcene, prepared from linaloöl by dehydration[25], excluding the following differences: absorptions at 1115 and 830 cm^{-1} were observed in myrcene, but they are absent in the tested specimen, also absorptions at 1168 and 735 cm^{-1} of this hydrocarbon are stronger than those of myrcene.

---

[22] S. Miyamoto and T. Ōkawa. "Toxic Plants for Domestic Animals", Kokuseido, Japan, 1942, p. 121.

[23] ethyl acetate was altered to ether in further experiments, so that we should avoid any trouble probable in identifying the ester components.

[24] all melting points are not corrected.

[25] B. A. Arbusow and W.S. Abramow, *Ber.*, **67**, 1942 (1934).

(II) Oxygenated Compounds

(A) Low boiling point fractions

(*1*) *Identification of ethyl alcohol:* An infrared spectrum of the compounds, boiling at 65–70°, indicates the presence of ethyl alcohol, ethyl acetate and methyl acetate. 3,5-Dinitrobenzoylchloride was added in excess to this fraction and reacted on a water bath for half an hour. The unreacted substance was distilled off. A white needle crystal, m.p. 92.5–93.5°, was obtained from the residue as in the usual method. Infrared spectrum of this crystal is identical with that of the authentic derivative of ethyl alcohol, the mixed melting point showing no depression.

(*2*) *Isolation of C_4 alcohol:* A fraction, boiling at 70–97°, was derived to 3,5-dinitrobenzoate. This derivative was spotted on a chromatostrip and developed with 15% ethyl acetate in *n*-hexane; two spots were observed by spraying 0.5% α-naphthylamine in ethyl alcohol. Therefore, the mixture was chromatographed on a silicic acid column by the method of White and Dryden[10]. A crystal was obtained after the solvent was evaporated off from the later portion of the effluent, both the melting point and infrared spectrum of this derivative being found identical with that of ethyl alcohol. Then, from the earlier portion of the effluent, a white needle crystal, m.p. 63–67°, was obtained, and an α-naphthylamine addition compound melted at 92.0–92.5°. According to elementary analysis, this crystal was supposed to be C_4 alcohol, but its chemical structure is not yet identified.

(*3*) *Identification of ethyl acetate:* A small amount of potassium hydroxide was added to the above mentioned fraction (1), separated from 3,5-dinitrobenzoate of ethyl alcohol by distillation, this reacting on a water bath for half an hour. After that, water was added, then it was heated ten minutes more. The reaction medium was saturated with sodium chloride and extracted with ether. The ether extract yielded a colorless liquid after the solvent was dried and distilled. 3,5-Dinitrobenzoate, derived from this extract, was identified as that of ethyl alcohol by its melting point, 90.9–91.6°, also by an agreement of infrared spectrum with that of authentic specimen. The chromatogram on the strip of its derivative showed only one predominant spot. Thus the presence of other alcohol is negligible. An alkali soluble part was acidified with sulfuric acid, saturated with sodium chloride. It was extracted with ether and the solvent was distilled off. The acid was derived to *p*-phenylphenacyl ester, m.p. 109.0–109.5°, which showed no depression of the melting point when mixed with the authentic acetic acid derivative, m.p. 111°. The infrared spectrum of the derivative was identical with that of acetic acid *p*-phenylphenacyl ester. The presence of other acids could not be estimated in this fraction.

(B) High boiling point fractions

(*1*) *Separation of an ester of benzoic acid:* After saponification of the eluate $(\mu)_6$, a crystal was obtained from the acid component of the ester. The melting point was somewhat low, 97–100°, the infrared spectrum was identical with that of benzoic acid.

(*2*) *Identification of α-pyrryl methyl ketone:* Removing the developing solvent from the eluate of $(\mu)_9$, a long white needle crystal was obtained. After several

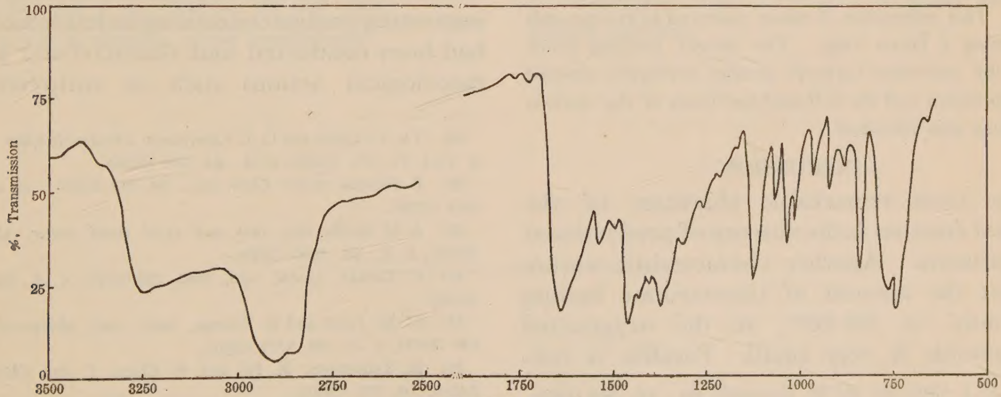


FIG. 2. The Infrared Absorption Spectrum of α-Pyrryl Methyl Ketone (a solid ground in Nujol).

recrysllizations from *n*-hexane, the melting point raised to 90.7–91.3°. *Anal.* Found: C, 64.82; H, 6.22; N, 12.70. Calcd. for C_6H_7ON: C, 66.03; H, 6.47; N, 12.84. When the crystal was developed with 15% ethyl acetate in *n*-hexane (v/v) on a chromatostrip besides by spraying the mixed acid mentioned above, a yellowish-orange spot, R_f value: 0.18, was observed. The infrared spectrum of this compound did not show a characteristic absorption near 1725–1706 cm^{-1} of simple ketone, caused by C=O streching vibration, although it formed crystalline 2,4-dinitrophenylhydrazone. Instead of this, a strong and somewhat broad absorption at 1642 cm^{-1} was observed which suggests the possibility of the presence of imino and ketonic linkages in very close position in a molecule. The mixed melting point with the authentic α-pyrryl methyl ketone[26] showed no depression, and both infrared spectra were in complete agreement with each other. 2,4-Dinitrophenylhydrazone was derived according to the usual method, but the recrystallization of its derivatives from common organic solvents was very difficult. After several recrystallizations from pyridine, containing a small amount of *n*-hexane, the melting point raised to 298° with decomposition identical with that of the authentic specimen. The infrared spectra of these derivatives were also found to agree completely with each other.

(*3*) *Identification of furfuryl alcohol:* The neutral compounds were directly saponified by the method of Redemann and Lucas[27]. From the alcoholic fraction, boiling at 100–200°, a crystalline 3,5-dinitrobenzoate, m.p. 80.0°, was obtained, closely resembling to that of furfuryl alcohol. On the two dimentional chromatostrip of the neutral compounds mentioned above, a purplish-gray spot appeared near the original position. This coloration is often observed in compounds possessing a furan ring. The mixed melting point with the authentic furfuryl alcohol derivative showed no depression and the infrared spectrum of the derivative was also identical.

DISCUSSION

The most remarkable character of the neutral fraction is the absence of predominant constituents. Another characteristic nature is that the amount of intermediate boiling fractions, ca 100–200°, in the oxygenated compounds is very small. Paraffin is considered to be mainly composed of C_{30} or C_{31}, but presence of other paraffins is probable. The infrared spectrum of the unsaturated hydrocarbon indicates that its structure might be as follows: i) this hydrocarbon belongs to the aliphatic; ii) it has either four or more than four methylenic linkages; iii) the presence of isopropyl or isopropylidene linkage is possible; iv) vinyl and vinylidene linkages might be present, and both double linkages are conjugated: v) another unsaturated linkage might be –HC=CH–; vi) few possibilities are estimated for the presence of the *trans* type –HC=CH– linkage. These estimations correspond to those from its molecular weight, specific gravity, refractive index and molecular refraction, which indicate that this hydrocarbon might have a close structure to myrcene, but it has an intermediate character between terpene and sesquiterpene. However, its chemical structure has not yet been completely clarified and further investigation is now in progress.

The presence of pyrrole has often been reported in tobacco essential oil and tobacco smoke[28,29,30], but so far no report can be seen concerning α-pyrryl methyl ketone. This compound had been found in valerian root[21,31,32] and in the essential oils of black tea[33]. In the case of valerian root, it was identified as an active principle of valeriana, interesting studies concerning its less toxicity[21] had been conducted and characteristic pharmacological actions such as antipyretic[34], anesthetic[35] and narcotic[19,20] actions to the nervous, especially respiratory and blood circulating systems. Furthermore, this compound is readily distillable, its taste was reported to be sweet and in no way a disagreeable pungent[18]. And then, it presents a neutral reaction with litmus and is not extractable with an aqueous solution of 3% sodium bisulfite. All these characteristic natures mentioned above considered to be caused by the chemical structure, which manifests that imino and ketonic linkages exist in a very close position in the molecule.

---

[26] G. L. Ciamician and M. Dennstedt, *Ber.*, **16**, 2348 (1883).

[27] C. E. Redemann and H. J. Lucas, *Ind. Eng. Chem. Anal. Ed.*, **15**, 521 (1937).

[28] Yu. O. Gabel and G. I. Kipriyanor, *Ukrainskii Khem. Zhur.* **8**, *Teck. Pt.*, 45, (1929); *C. A.*, **24**, 916 (1930).

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[33] R. Yamamoto, K. Itō and H. Chien, *J. Agr. Chem. Soc. Japan*, **16**, 800 (1940).

[34] A. Rabbeno, *Arch. intern. pharmacodynamie*, **36**, 387 (1930); *C. A.*, **25**, 3397 (1931).

Recently, Frankenburg and et al.[36] have reported the presence of 3-pyridyl propyl ketone in the extracts of fermented cigar tobacco leaves as a degradation product of nicotine. Both compounds, 3-pyridyl propyl ketone and α-pyrryl methyl ketone, have many common structural natures, but the physiological and chemical natures of these compounds seem to differ much in the respect that the former has a pyridine nucleus, with the side chain attached at the β-position, while on the contrary, the latter has a pyrrole nucleus with the side chain attached at the α-position.

Tinctures of valerian root have been frequently used for tobacco flavor, especially for cigar and snuff[37]. We have heard that it has been practically used for the purpose of cigarette flavor in some countries, in such a high concentration as 40–50% for long years. The reason of this usage is chiefly attributed to its specific activity in making tobacco smoke mild thus improving its "Smoking quality". However, the principal activity of this favorable character had not yet been clarified. We confirmed that those activities mentioned above are fully induced by α-pyrryl methyl ketone which has no pungent, no irritating, no harshy, but a favorable aromatic, agreeable taste, narcotic, anesthetic, less toxic and easily distillable features.

We wish to express our cordial thanks to Prof. Dr. Y. Sumiki, Department of Agricultural Chemistry, University of Tokyo for his wise direction and kind guidance, also to Director T. Nakashima, of the Central Research Institute, Japan Monopoly Corporation for his strong support to us. We are indebted to Dr. M. Matsui, Department of Agricultural Chemistry, University of Tokyo, for the microanalysis and Mr. T. Miyazawa, Department of Chemistry, University of Tokyo, for the infrared analysis and Miss Y. Iwase, Mr. K. Saito for their cooperation in carrying out this experiment. Last but not least, we are greatly indebted to Emeritus Prof. Dr. T. Yabuta for his sound advice.

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[35] A. Rabbeno, *Boll. soc. ital. biol. sper.*, **5**, 1094 (1930); *C. A.*, **25**, 2484 (1931).

[36] W. G. Frankenburg, A. M. Gorrscho, A, A. Veitekunas and R. M. Zachrrius, *J. Am, Chem. Soc.*, **77**, 5730 (1955).

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Studies on the Essential Oils of Tobacco Leaves

Part V. The Isolation of β-Methylvaleric Acid and 2-Furoic Acid from Tobacco Essential Oil

By Isao ŌNISHI and Kyoji YAMASAKI

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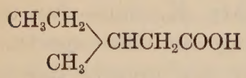
We have isolated and identified β-methylvaleric acid and 2-furoic acid from the steam distillable components, "Essential Oil", of fermented Virginia tobacco leaves.

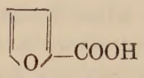
Previously[1], we have reported the isolation and identification of formic, acetic, isobutyric, *trans*-crotonic, *n*-caproic, benzoic and phenylacetic acid, the isolation of a fatty acid, preliminarily estimated to be a pentoic acid from the "Acid Fraction". Afterwards, we have identified the pentoic acid as β-methylvaleric acid (I).

By estimating the "Hydrolyzed Acid Fraction" (see Table I of the previous paper[1]), which was obtained by hydrolysis of esters contained in the tobacco essential oil in the course of separation into each fraction, formic, acetic, benzoic and 2-furoic acids (II) have been isolated and identified by the same procedure employing chromatography and infrared spectra as reported previously[1].



$\left.\begin{matrix}CH_3CH_2\\CH_3\end{matrix}\right\rangle CHCH_2COOH$

I. β-Methylvaleric acid.



II· 2-Furoic acid.

EXPERIMENTAL

(1) Identification of β-Methylvaleric Acid: Acid II-1 and Acid I-2 of the previous paper[1] were isolated by column chromatography using silicic acid as the packing and iso-octane as the developer. The melting point of the *p*-bromophenacyl ester of these acids agreed with the derivative of β-methylvaleric acid isolated by Sabetay and Panouse[2] from tobacco leaf, but the microanalysis values showed that of a pentoic acid. Afterwards we prepared β-methylvaleric acid (b.p. 193–6°/760 mm.) according to the method described in Organic Synthesis[3]. *p*-Bromophenacyl esters of Acid II-1 and Acid I-2 showed no depression when mixed with the authentic derivative of β-methylvaleric acid and infrared spectra of both derivatives agreed well with each other (Fig. I)[4].

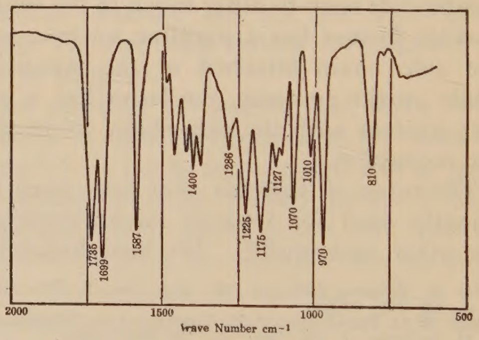


FIG. I. Infrared Spectrum of β-Methylvaleric acid *p*-Brom phenacyl ester. (Nujol paste.)

(2) Preparation of "Hydrolyzed Acid Fraction": We obtained the "Hydrolyzed Acid Fraction" (47.878 g) from flue-cured Virginia Bright Yellow type tobacco leaves, harvested in Okayama district in 1952 (136 kg) by the method described in the previous paper[1]. This fraction contained a considerable amount of dark colored resinous substances. In order to remove the impurities the fraction was extracted with hot iso-octane, the iso-octane soluble fraction (yield 13.571 g) being used to separate organic acids by column chromatography. A suitable amount of the "Hydrolyzed Acid Fraction" was placed on a column (400 × 18 mm. dia.) of silicic acid using iso-octane as the developer according to Vandenheuvel and Hayes's method[5]. Each organic acid isolated by column chromatography was derived to its *p*-bromophenacyl ester, then identified by its melting point, microanalysis and infrared spectrum.

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[1] I. Ōnishi and K. Yamasaki, This Bulletin, **19**, 137 (1955).

[2] S. Sabetay and J. Panouse, *Compt rend.*, **25**, 887 (1947).

[3] Organic Syn. Coll. Vol. II, p. 416.

[4] Infrared spectra of the derivatives were obtained with a Perkin-Elmer Model 21 infrared spectrophotometer installed in our Research Institute.

(3) **Identification of 2-Furoic Acid:** It was estimated that the "Hydrolyzed Acid Fraction" contained a considerable amount of acetic and formic acids with a small amount of benzoic acid. To remove these low molecular fatty acids, the "Hydrolyzed Acid Fraction" was distilled at atomospheric pressure (to bath temp. 160°). The residue of distillation was dissolved in ether, the ethereal solution was extracted with a saturated aqueous solution of sodiumbicarbonate. After this extract was acidified with 80% sulfuric acid, saturated with sodium chloride, and extracted with ether, the ethereal solution was dried over anhydrous sodium sulfate. After removal of ether, a small amount of white crystals was obtained and recrystallized from boiling water (yield 18 mg, m.p. 128–30°). This crystal showed no depression of melting point when mixed with the authentic 2-furoic acid (m.p. 132–3°), prepared by the method described in Organic Synthesis[6]; the infrared spectra were found to agree completely with each other. *Anal.* Found: C, 52.54; H, 3.68, Calcd. for $C_5H_4O_3$: C, 52.58; H, 3.60.

CONCLUSION

β-Methylvaleric acid was isolated and identified from Essential Oil of flue-cured Virginia tobacco leaf. This acid was already isolated from Algeria tobacco leaf by Sabetay and Panouse. As reported in the previous paper, β-methylvaleric acid was a main component (20% for total acid)[1] of the "Acid Fraction", together with acetic acid. This acid has a somewhat sweet acidic odor, better than *n*-caproic acid, but less than iso- and *n*-valeric acids, and it might be responsible for the aroma and taste of tobacco leaves. It was intersting that we could not isolate *n*- and iso- valeric acids, and it have been frequently reported to be contained in flue-cured Virginia tobacco leaves.

The fact that formic, acetic, benzoic and 2-furoic acids were isolated from the "Hydrolyzed Acid Fraction" might suggest the presence of esters of these acids in the "Neutral Fraction". This presumption was supported by the isolation and identification of ethyl acetate and a benzoate from the "Neutral Fraction"[7].

We wish to express our cordial thanks to Prof. Dr. Y. Sumiki, Department of Agricultural Chemistry, University of Tokyo, for his wise direction and kind guidance, and to Director T. Nakashima, Central Research Institute, Japan Monopoly Corporation, for his strong support to us. We are also indebted to Dr. M. Matsui, Department of Agricultural Chemistry, University of Tokyo, for the microanalysis and Miss H. Ōtuka, Mr. K. Saitō for their cooperation in carrying out this experiment. Last but not least, we are greatly indebted to Emeritus Prof. Dr. T. Yabuta for his sound advice.

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[5] F. A. Vandenheuvel and E. R. Hayes, *Anal. Chem.*, **24**, 960 (1952).

[6] Organic Syn., Coll. Vol. I, p. 276.

[7] I. Ōnishi, H. Tomita and T. Fukuzumi, This Bulletin, **20**, 61 (1956).

[Bull. Agr. Chem. Soc. Japan, Vol. 20, No. 2, 70~73 pp, 1956]

Studies on the Essential Oils of Tobacco Leaves

Part VI. Phenol Fraction (II)

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Further investigation was conducted on the caustic alkali soluble compounds contained in the essential oil of Virginia tobacco leaves. Benzaldehyde, *p*-anisaldehyde, *o*-hydroxyacetophenone and eugenol were isolated and identified. Several other compounds were also isolated and investigated.

In the previous paper¹⁾, we have described isolation and identification procedures of phenol, guaiacol, salicylaldehyde, methyl salicylate, lauric acid, myristic acid and palmitic acid, also presuming the presence of several carbonyl compounds and a low boiling substance. In the course of further investigations other two phenolic compounds were detected by paper chromatography in the alkali insoluble part, which was separated when the crude phenol ethereal solution was shaken again with an aqueous solution of 3% potassium hydroxide for purification. In this paper, we have identified eugenol as one of these phenolic compounds, by fractional distillation followed by chromatography. The final identification was accomplished on melting point, and infrared spectrum of its 2, 4-dinitrophenylether. The carbonyl compounds were further separated and derived to 2, 4-dinitrophenylhydrazones. Acetaldehyde, benzaldehyde, *p*-anisaldehyde, *o*-hydroxyacetophenone as well as salicylaldehyde were isolated and identified by chromatographic adsorptions on silicic acid and celite, then, finally by their melting points, infrared spectra and elementary analyses. On the other hand, the presence of *m*-tolualdehyde was estimated. Heretofore, no report concerning *p*-anisaldehyde, *o*-hydroxyacetophenone and *m*-tolualdehyde in the essential oil of tobacco leaves can be seen. So far as the presence of eugenol is concerned, Piatniski²⁾ has once reported on this, but his details had not been clarified. However, its presence has been clearly confirmed in this study.

EXPERIMENTAL

In order to purify an ethereal solution of crude phenol, it was shaken again with an aqueous solution of 3% potassium hydroxide, and a brown oil remained on evaporating off the ether. The residual oil solidified and separated white crystals on standing (ca. 1.2 g.). As it slightly showed a positive diazo reaction, it was dissolved in 20 ml. ether and poured into the 210×25 mm. alumina (Brockman) chromatographic column. It was eluted by ether for the first time, followed by ethyl acetate.

1. A yellow oil (20 mg.) remained on distilling off the ether effluent. Its 2, 4-dinitrophenylhydrazone is hardly soluble in hot alcohol and a black particle was obtained by recrystallization from alcohol-ethyl acetate.

2. An orange-yellow oil (10 mg.) was obtained from the ethyl acetate effluent. Two spots, R_F values: 0.35, pink and 0.55, yellow, were detected on paper chromatograms of its hydroxyazo compounds. Though these spots were not identical with the previously detected compounds¹⁾, one of them, R_F value: 0.55, was concordant with that which we detected in the volatile phenol of the "Freon" extracts of Virginia

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1) I. Ōnishi and K. Yamamoto, This Bulletin, **19**, 148 (1955).

2) W.G. Frankenburg. *Advances in Enzymology*, **6**, 331 (1946).

tobacco leaves[3].

3. The column was separated into six portions with equal length, each portion, after acidification was extracted with ether. A yellowish-white solid substance, 1.020 g., was obtained from the upper portion. On recrystallizing from 75% alcohol, palmitic acid was identified by its melting point, 61–62°, and infrared spectrum. These compounds seemed to remain in the ether because of their incompleteness of separation. It was evident that at least two unknown phenols, and certain amounts of carbonyl compounds were presented in this fraction, so we have conducted investigation separately on carbonyl and phenol fractions.

Carbonyl Fraction: Crude phenol ether solution, which was obtained from 120 kg. Virginia tobacco leaves by the same method as described in Part III, was shaken again with an aqueous solution of 20% sodium bisulfite to separate the carbonyls. After being washed with ether, added with calculated 6 N sulfuric acid, saturated with sodium chloride, the aqueous solution was repeatedly extracted with ether. As the ethereal solution colored dark on distilling off the ether, 20 ml. alcohol was added just before the ether distilled up, this being poured into the solution consisting of 0.5 g. 2, 4-dinitrophenylhydrazine, 2 ml. concentrated sulfuric acid, 3 ml. water and 40 ml. alcohol. After thorough stirring, a separated reddish-brown precipitate of 2, 4-dinitrophenylhydrazone (I), 169 mg., was filtered off, then it was washed and dried. On the other hand, an orange-yellow precipitate of 2, 4-dinitrophenylhydrazone (II), 76 mg., was obtained from the filtrate.

TABLE I

CHROMATOGRAPHIC SEPARATION OF THE MIXTURE OF 2, 4-DINITROPHENYLHYDRAZONES (I) FROM THE CARBONYL FRACTION (sample 169 mg.)

Band No.	Yield (mg.)	Compound	Color
5	115	(α-Pyrryl methyl ketone)	Reddish-black
4	28	Salicylaldehyde	Red
	12	*o*-Hydroxyacetophenone	Orange
3	1	*p*-Anisaldehyde	Red
2	5	Benzaldehyde	Orange-yellow
1	4	(*m*-Tolualdehyde)	Red

Band number in order of elution.
Adsorbent: Silicic acid+Celite (2:1).
Column: 200×36 mm.
Developing solvent: 1–10% Ether in petroleum benzine (b.p. 40–80°).

Chromatographic Separation of (I): Forty milligrams of (I) was put into the chromatographic column by the same method as described in Part II. of this study. The same procedure was repeated four times, the results obtained are shown in Table I. The colored effluents of Bands No. 1 and 2. were collected in each flask successively while the other bands were sectioned, then extracted with ether. On distilling off the solvent, a dry precipitate remained in each flask.

Estimation of m-*tolualdehyde:* Recrystallization of the precipitate from Band No. 1 gave a red crystal, sublimizing at 193–197°. Its infrared spectrum is similar to 2, 4-dinitrophenylhydrazone of *m*-tolualdehyde, m.p. 200–201°, but not decidedly identical. Further investigation is now in progress.

Identification of benzaldehyde: An orange-yellow crystal, m.p. 240–241°, was obtained from Band No. 2. The mixed melting point with the authentic derivative of benzaldehyde, m.p. 243°, did not depress. Infrared spectra were found to agree well with each other.

Identification of p-*anisaldehyde:* A red plate crystal, m.p. 254°, was obtained from Band No. 3. by recrystallization from 95% alcohol. The melting point did not depress by mixing with the authentic derivative of *p*-anisaldehyde, m.p. 253–254°. The infrared spectra were found to agree well with each other.

Identification of salicylaldehyde and o-*hydroxyacetophenone:* Band No. 4 which developed just beneath the top band, was separated into two bands by repeated chromatography employing 0–10% ethyl acetate in petroleum benzine. Although the separation was not complete, finally, the mainly colored portions were extruded, purified by the same chromatographic procedure and recrystallized from the alcohol-ethyl acetate mixture. A fine red needle crystal, m.p. 252°, was obtained from the upper band. The mixed melting point with the authentic derivative of salicylaldehyde did not depress, both infrared spectra were found to be identical with each other. From the lower band, a fine orange needle crystal, m.p. 210°, was obtained. *Anal.* Found: C, 53.24; H, 3.84; N, 17.65, Calcd. for $C_{14}H_{12}O_5N_4$: C, 53.15; H, 3.76; N, 17.72. The infrared spectrum of this crystal was identical with 2, 4-dinitrophenylhydrazone of the authentic *o*-hydroxyacetophenone[4].

Estimation of α-pyrryl methyl ketone: The top band did not descend entirely and was hardly soluble in hot alcohol. It was therefore extracted with ether and a black particle was obtained after removing the solvent and recrystallizing from alcohol and ethyl acetate. Although we could not determine the melting point, this substance is similar to that of the component isolated from Fraction 4, Table I., in the previous paper[1], the infrared spectrum was identical with 2, 4-dinitrophenylhydrazone of α-pyrryl methyl ketone, found in the neutral fraction of this study.

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[3] I. Ōnishi and K. Yamamoto, *Scientific Papers of Central Research Institute, Japan Monopoly Corporation*, No. **90**, 39 (1954).

[4] K. Fries and W. Phaffendorf, *Ber.*, **43**, 215 (1910).

Chromatographic Separation of (II): The results are summarized in Table II.

TABLE II

CHROMATOGRAPHIC SEPARATION OF THE MIXTURE OF 2, 4-DINITROPHENYLHYDRAZONES (II) FROM THE CARBONYL FRACTION (sample 76 mg.)

Band No.	Yield (mg.)	Compound	Color
4	25	Unknown	Reddish-black
3	42	Acetaldehyde	Orange
2	less than 1	Unknown	Yellow
1	3	Unknown	Orange

Foot notes are the same as in Table I.

Band No. 1: A fine orange needle crystal obtained from Band No. 1. was recrystallized twice from 94% alcohol, m.p. 134°. We cannot find any identical 2, 4-dinitrophenylhydrazone with this in the literature, so further investigation is now in progress.

Band No. 2: We could not measure neither the melting point nor the infrared spectrum because the amount was too small.

Identification of acetaldehyde: From Band No. 3, an orange needle crystal was obtained, recrystallized from 90% alcohol, m.p. 162°. The mixed melting point with the authentic acetaldehyde 2, 4-dinitrophenylhydrazone, m.p. 164°, showed no depression and the infrared spectra of both derivatives agreed well with each other.

Band No. 4: The top band was similar to Band No. 5. in Table I.

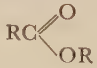
Phenol Fraction: After removal of the carbonyl compounds, crude phenol, 19.6251 g., was put into the fractional distillation under a nitrogen stream. The results are shown in Table III. Fraction 1. was almost ether. Fraction 2. was also almost ether, but it seemed to contain some other compounds. Two peculiar infrared absorption bands, 1730 and 1230 cm^{-1}, which were absent in the authentic ether, were found in this fraction. As these bands indicated the presence of C=O linkage in the form of  $RC\langle^{O}_{OR}$, and especially from the latter band attributed to acetate, the presence of a low molecular ester was presumed, but we could not identify any component after saponification because of the lack of sufficient amount for further investigation.

TABLE III

SEPARATION OF THE CRUDE PHENOLS BY FRACTIONAL DISTILLATION (sample 19.6251 g.)

Fraction	Pr.	b.p. (°C)	Bath T. (°C)	Yield (g.)
1		(~ 35)	83	1.2032
2		(~ 45)	125–145	0.8650
3	6 mm. (Hg.)	(~ 57)	180–200	0.1265
4	〃	(~160)	236	1.2016
Residue				13.3201

Detection of phenol and guaiacol: Two spots were detected on the paper chromatogram of Fraction 3. They were indentical with phenol and guaiacol both in their R_F values and colors, according to our previous paper[1].

Identification of eugenol: Fraction 4, a mixture of phenolic compounds and high molecular fatty acids, was put into the liquid chromatography as follows: the mixture was dissolved in 20 ml. of ether then poured into the chromatographic column of silicic acid (230×30 mm.). On eluting with petroleum benzine, b.p. 30–65°, a fatty acid, 1.006 g., appeared in the first effluent. Thirty-one milligram of orange-yellow oil was obtained from the ethyl acetate effluent. One spot, which corresponds to R_F value: 0.35, pink, described above, was detected on the paper chromatogram. This oil was derived to 2, 4-dinitrophenylether according to the method of Bost and et al.[5]. A yellow plate precipitate, 7 mg., was obtained by final recrystallization from alcohol. The mixed melting point of this derivative, m.p. 112–113°, with the authentic derivative of eugenol, m.p. 114°, did not depress, and the infrared spectrum was identical with that of the authentic specimen. The fact that eugenol was not identified from the corresponding fraction in the previous experiment might be attributable to the quantitative loss by purification and the mixture containing a large amount of fatty acids. In such a case it is hard to detect the phenols by any method. Another phenol corresponding to R_F value: 0.55, was not identified in this investigation.

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[5] R.W. Bost and F. Nicholson, *J. Am. Chem. Soc.*, **57**, 2368 (1935).

DISCUSSION

From the phenolic fraction of Virginia tobacco essential oil the following substances were isolated and identified: phenol, guaiacol, eugenol, *p*-anisaldehyde, *o*-hydroxyacetophenone, benzaldehyde, acetaldehyde, salicylaldehyde and palmitic acid. The presence of α-pyrryl methyl ketone was also proved in this fraction. Another compound such as *m*-tolualdehyde was isolated but could not be decidedly identified.

Among these compounds, phenol, guaiacol, salicylaldehyde and palmitic acid have already been reported in the previous paper1). Actaldehyde and benzaldehyde were also reported in this study6). Heretofore, we have no report indicating the presence of *p*-anisaldehyde, *o*-hydroxyacetophenone, *m*-tolualdehyde and α-pyrryl methyl ketone in tobacco essential oils. Eugenol has been reported by other authors2), but the method of identification was not so distinct such as in this study.

It is an interesting fact that α-pyrryl methyl ketone, which was found in the neutral fraction described in the previous paper7), was also found in this phenol fraction. As mentioned above, many carbonyls and some acidic compounds were isolated besides the true phenolic compounds from this fraction. The isolation of phenols seems to be difficult, and the solubility in aqueous and ethereal solutions has influence on the partition of these chemically inactive compounds, especially when the amount is very small.

Although we could not determine accurate quantity, the amount of palmitic acid far exceeds the others. It is well known that high molecular fatty acids have the property making tobacco smoke mild. On the other hand, undoubtedly, each of the other substances mentioned above also have their characteristic intense odor. So, it is clear that they have influence upon the aromas and tastes of tobacco smoke, either individually or together, though the amounts are small.

We wish to express our cordial thanks to Prof. Dr.Y. Sumiki, Department of Agricultural Chemistry, University of Tokyo, for his wise direction and kind guidance, and to Director T. Nakashima, Central Research Institute, Japan Monopoly Corporation, for his strong support to us. We are also indebted to Dr. M. Matsui,Department of Agricultural Chemistry, University of Tokyo, for the microanalysis and Mr. H. Takahara, Mr. K. Saitō for their cooperation in carrying out this experiment. Last but not least, we are greatly indebted to Emeritus Prof. Dr. T. Yabuta for his sound advice.

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6) I. Ōnishi and M. Nagasawa, This Bulletin, **19**, 143 (1955).

7) I. Ōnishi, H. Tomita and T. Fukuzumi, This Bulletin, **20**, 67, (1956).

[Bull. Agr. Chem. Soc. Japan, Vol. 20, No. 2, 74~76 pp, 1956]

Studies on Cycasin, a New Toxic Glycoside, of *Cycas revoluta* Thunb

Part V.* Quantitative Determination of Cycasin in Cycad Seeds

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Quantitative paper chromatography of cycasin is described, where the cycasin and sugars separated on the paper are eluted out and determined, according to the colorimetric micro analysis of sugars by Plumel. The contents of cycasin and free sugars contained in both premature and matured cycad seeds determined by this method are presented.

In the preceding papers[1] it was reported that cycasin is easily hydrolyzed with acid and especially with alkali and in this case its aglycone also decomposes immediately into lower molecular compounds. The determination of cycasin, therefore, should be conducted either on the liberated glucose or on these degradation products. As a relatively specific one among the latter method, the cyanide in the alkali decomposition can be used for qualitative detection of cycasin, as reported in Paper III of this series. It is, however, difficult to apply this for quantitative purposes since neither its stoichiometric production nor sensitive reaction is yet available. Although the products of the acid hydrolysis, for example formaldehyde, are obtained in one mole ratios per one mole of cycasin, further studies are necessary for determination of these compounds because of the several difficulties encountered such as those coexisting interfering substances. In the case of utilizing the reaction with the sugar component, the chromatography resolves not only the question of separating cycasin from the coexisting sugars but also is advantageous in the simultaneous determination of these sugars.

In this paper, a quantitative paper chromatography and a colorimetry based upon the reducing power determination of the sugars were adopted as a simple-rapid method for determination of cycasin and sugars in the cycad seeds. Although various devices have been made on quantitative paper chromatography, it is considered to be appropriate for the present purpose that the spots on the paper, developed one-dimensionally, are eluted and determined colorimetrically. After examinations of several colorimetries for sugars applicable to cycasin, Plumel's method[2] was proved to be most suitable; by this method, micro amounts of the sample are measurable and it is convenient in regard to the reagents and procedure.

This method is based on the reduction of potassium ferricyanide with reducing sugars and the color of prussian blue formed by the addition of ferric alum. It was learned that the non-reducing sugar, sucrose, can also be determined and that the inverting condition gives the maximum coloration. The appropriate time for the reduction of ferricyanide, that is the "heat period" in a boiling-water bath, is not the same for cycasin and each sugar. The colors produced by

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* Parts III and IV are presented in the *J. Jap. Biochem. Soc.*

[1] K. Nishida, A. Kobayashi and T. Nagahama, This Bulletin **19**, 77, 172 (1955).

[2] M. Plumel, *Ann. biol, chim.*, **9**, 307 (1951), *C. A.*, **47**, 11082 (1953).

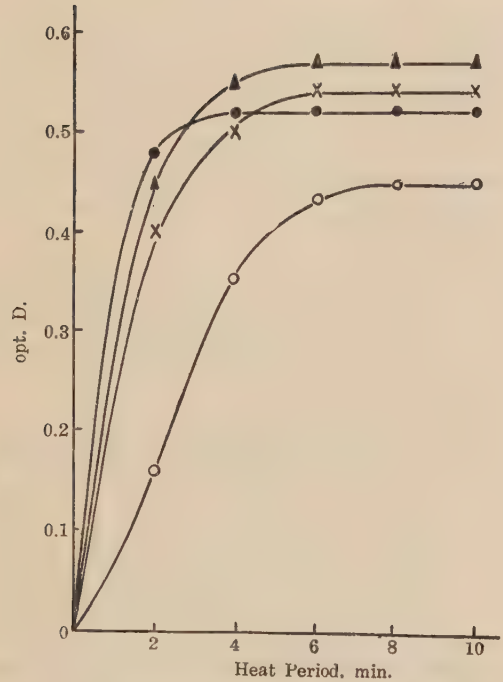

FIG. 1. Relation between Heat Period and Coloration.

○, cycasin. ●, fructose. ×, glucose. ▲, sucrose.

100γ of cycasin, fructose, glucose and sucrose* (*means inverted) heated at various periods are shown in Figure 1. Fructose reduces ferricyanide most rapidly, and glucose and sucrose* almost similarily. From the curve of cycasin it is observed that there is a time-delay where glucose is liberated despite of the extreme instability of cycasin in alkaline solution. From these data, the heat period most appropriate was decided as to be twelve minutes for cycasin, ten minutes for glucose and sucrose*, and eight minutes for fructose. The standard curves for cycasin and these three sugars are shown in Figure 2, which in passing the zero point, show fair conformation to Beer's law.

Chromatography was carried out in the usual manner and the spots were eluted according to the method of Nakabayashi[3] which was found to be most advantageous for a number of samples. The appropriate amount separable on paper is about 50γ, which agrees with the suitable colorimetric range presented by the prussian blue method. Therefore, the throughout process of this determination can be executed conveniently and rapidly. Recovery of the specimen by paper chromatographic determination, is presented in Table I. Although errors may occur at times on fructose and glucose whose spots are located adjacent on the chromatograms, the method described here on the whole gives satisfactory results.

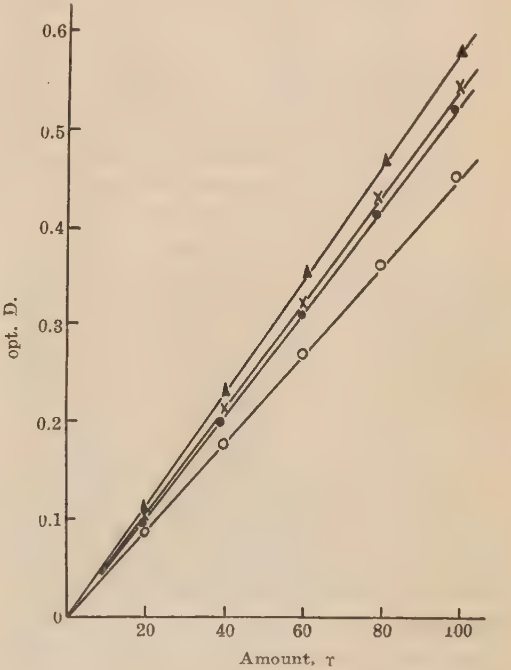

FIG. 2. Standard Curves for Cycasin and Sugars. The Symbols are same as in FIG. 1.

In the southernmost region of Japan where cycad grows wildly, the flower season is around July, and the gathering of matured seeds begins about October. The seeds sampled on July 31. and October 10. were, therefore, made as two examples representing both the premature and matured stages, respectively. From the results of determining the contents of cycasin and sugars in the

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[3] T. Nakabayashi, *J. Agr. Chem. Soc. Japan*, **27**, 272 (1953).

TABLE I

RECOVERY OF CYCASIN AND SUGARS

Sugars	Calcd.	Found	Recovery	⊿
Cycasin	61.0γ	60.9γ	99.9%	−0.1%
	61.2	63.5	103.8	+3.8
	61.6	62.8	102.0	+2.0
Fructose	59.0	60.0	101.6	+1.6
	59.1	61.5	104.1	+4.1
	59.5	61.5	103.3	+3.3
Glucose	59.0	60.2	102.0	+2.0
	59.1	58.5	99.0	−1.0
	59.5	60.9	100.9	+0.9
Sucrose	56.6	59.2	104.6	+4.6
	56.8	58.5	103.0	+3.0
	57.3	58.6	102.1	+2.1

TABLE II

ANALYSES OF CYCASIN AND SUGARS IN CYCAD SEEDS

Date of Sampling	July 31, 1955	Oct. 10, 1955
Moisture	87.37%	46.84%
Wt. of one fresh kernel	7.1 g	10.9 g
Cycasin	0.28%	0.17%
Fructose	0.44	0.04
Glucose	0.24	0.26
Sucrose	0.69	1.93

Contents are represented as per cent in fresh kernel.

extracts of these sample seeds, some interesting facts were learned. As shown in Table II, the cycasin contents decreased slightly after maturing. The contents of glucose remained almost constant, while fructose decreased remarkably in contrast with the increase of sucrose.

MATERIALS AND METHODS

Paper Chromatography. Errors caused by the conditions of paper were made negligible by the use of Toyo No. 131 Filter Paper which was washed twice preliminarily with distilled water in the ascending method. In addition, control experiments on the developed paper, that is, the correction for the adsorption of the eluate from the paper-blank (optical density ca. 0.02) were conducted.

An aqueous sample solution, of 20 to 30μl, was spotted with a micropipette on the original line of the paper at a 2-cm interval, and spots were developed at about a 30-cm distance from the original line with a *n*-butanol-aceticacid-water mixture (4 : 1 : 1), by one-dimensional ascending method. After air-drying the solvent, the positions corresponding to the spots of cycasin and sugars were determined according to the guide strips which were revealed by spraying with resorcin or diphenylamine reagent. Though elution of the cut pieces of paper (20×18∼24 mm) was achieved even with 0.1 ml of distilled water, 2 to 3 ml of the eluate was taken for convenience of colorimetry.

Condition of Inversion. The inverting condition for sucrose which gives the maximum coloration was decided as follows.

Five ml of the sample solution containing 1 to 100γ of sucrose was added with 0.5 ml of N/10-hydrochloric acid, heated in a boiling-water bath for twenty minutes, then neutralized with 0.5 ml of N/10-sodium hydroxide solution.

Colorimetry. To 5 ml of sample solution in a test tube, containing 1 to 100γ of cycasin or sugar, 1 ml of a ferricyanide solution (potassium ferricyanide 0.66 g, potassinm ferrocyanide 0.02 g, anhydrous sodium carbonate 1.06 g and water to 100 ml) was next added and heated in a boiling-water bath for a definite period. After cooling with tap water, 1 ml of a fluoride solution (sodium fluoride 1.26 g, sodium acetate 1.36 g and water to 100 ml) and 2 ml of an alum solution (ferric alum 1.2 g, N-sulfuric acid 13.75 ml and water to 100 ml) was added. It was diluted to 25 ml; measured by colorimetry with a Shimadzu Photoelectric tube colorimeter using filter S_{57} and a cuvette of 10 mm.

The coloration of prussian blue reaches its maximum degree fifteen minutes after addition of the alum solution and is stable for two to three hours, but precipitates thereafter.

Preparation of Sample from Cycad Seeds. Samples were collected on July 31, and October 10, 1955 at the suburbs of Nasé City, Kagoshima Prefecture. Eighteen seeds, 276.5 g, collected on July were hulled carefully and the kernels, 119.6 g, were thrown immediately into 200 ml of boiling methanol, and refluxed for twenty minutes. After mashing in a mortar, it was boiled again with additional 100 ml of water for thirty minutes and filtered. Extraction of the residues with 200 ml of 50% hot methanol was repeated three times more. The combined extracts was treated with neutral lead acetate solution and hydrogen sulfide gas as usual, and condensed in vacuum below 50°C to 25 ml, then made as the stock solution. For the sample collected on October, four seeds, weighing 66.6 g, were similarily treated with minor volumes of methanol, and 10 ml of stock solution was obtained. Paper chromatography was carried out with these stock solutions, after proper dilution.

[Bull. Agr. Chem. Soc. Japan, Vol. 20, No. 2, 77~79 pp, 1956]

Studies on Synthetic Pyrethroids

Part VIII. Addition of Ethyl Diazoacetate to Methyl α, β, δ-Trimethylsorbate

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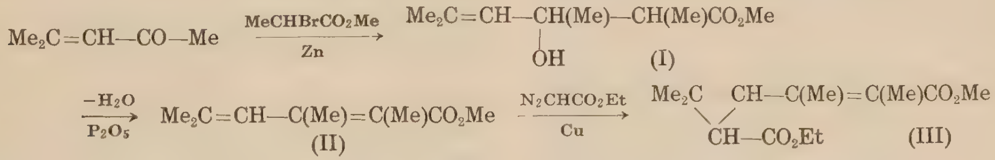
Received March 13, 1956

Ethyl diazoacetate adds predominantly to the $\gamma\delta$-ethylenic bond of methyl α, β, δ-trimethylsorbate to yield the cyclopropane derivatives, which are shown by the isolation of (±)-*cis*-, and (±)-*trans*-caronic acids from ozonolysis and subsequent hypobromite oxidation of the adducts. One of the adduct acid, obtained crystalline m.p. 164–5°, is shown to be (±)-*cis*-3-(1′-methyl-2′-carboxypropenyl)-2, 2-dimethylcyclopropane-1-carboxylic acid both by spectroscopic and degrative evidence.

Prior to the performance of the addition of the ethyl diazoacetate to ethyl α, δ-dimethylsorbate[1], it was necessary to know which of the two ethylenic bonds present in the sorbate molecule would be attacked by diazoacetate, and also what reaction conditions should be employed for successful $\gamma\delta$-addition to yield chrysanthemum-dicarboxylic acid. For this purpose, model reactions were carried out on methyl α, β, δ-trimethylsorbate, which had a structure close to α, δ-dimethylsorbate and though hitherto undescribed, might be prepared from the Reformatski condensation of readily accessible mesityloxide with methyl α-bromopropionate. Furthermore, the $\gamma\delta$-addition of diazoacetate to this model compound could be easily indicated by isolation of the known caronic acids from the oxidation products of the adducts, and the absence of geometrical isomerism with respect to the $\gamma\delta$-ethylenic bond in this molecule would facilitate stereochemical consideration of the products.



$$Me_2C{=}CH{-}CO{-}Me \xrightarrow[Zn]{MeCHBrCO_2Me} Me_2C{=}CH{-}\underset{OH}{CH}(Me){-}CH(Me)CO_2Me \quad (I)$$

$$\xrightarrow[P_2O_5]{-H_2O} Me_2C{=}CH{-}C(Me){=}C(Me)CO_2Me \quad (II) \xrightarrow[Cu]{N_2CHCO_2Et} \quad (III)$$

The experiments described here had been carried out during 1953–1954, prior to the investigation already published in Part V.

Concerning the addition of aliphatic diazo-compounds to unsymmetrical diene systems substituted by polar groups, the experiments available were insufficient for any confident prediction to be made in regard to this point. However, theoretical consideration and some reactions relating to the conjugated diene systems, as was briefly mentioned in Part V, seemed to be in favour of the $\gamma\delta$-addition.

After unsuccessful attempts to effect the $\gamma\delta$-addition and to isolate the crystalline cyclopropane adducts, ethyl diazoacetate was added to methyl α, β, δ-trimethylsorbate in boiling ligroin, in the presence of copper powder, following D'yakonov's procedure[2].

Hydrolysis of the adduct ester gave a viscous acid which partially crystallised after a long standing in the cold. Recrystallisa-

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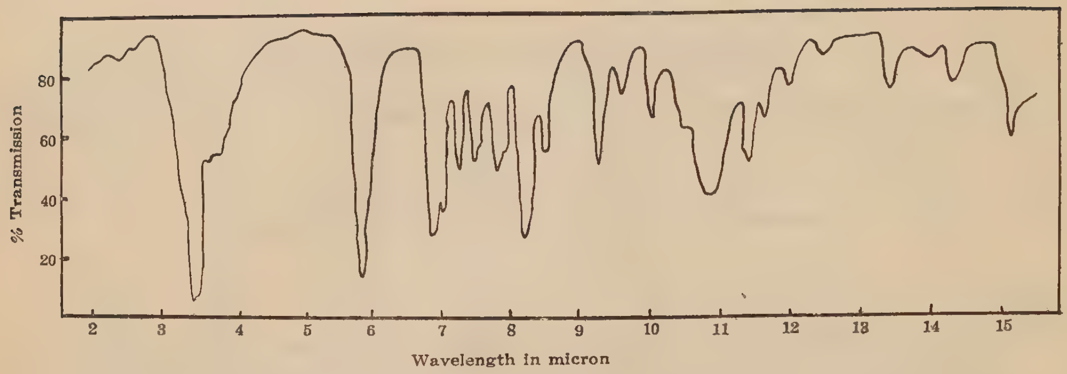
[1] Y. Inouye, Y. Takeshita and M. Ohno; This Bulletin, **19**, 193 (1955): *Botyu Kagaku*, **20**, 102 (1955).



Infrared Spectrum of m.p. 164–5° Acid in Nujol.

tion of the solid gave a dibasic acid $C_{11}H_{16}O_4$, m.p. 164–5°, which was also characterised by di-*p*-phenylphenacylester.

Ultra-violet light absorption spectrum (λ max 238 mμ, ε 19,700) indicated the presence of the same chromophore system in this molecule as that in chrysanthemum-dicarboxylic acid (see Part V).

The infrared absorption spectrum (Fig.) was very similar to that of chrysanthemum-dicarboxylic acid (*cf.* Part V), the occurrence of the band at 10 μ indicating the presence of the cyclopropane ring. In addition to these evidences, the isolation of *cis*-caronic acid from ozonolysis and subsequent hypobromite oxidation of the acid revealed the structure to be (±)-*cis*-3-(1′-methyl-2′-carboxypropenyl)-2, 2-dimethylcyclopropane-1-carboxylic acid, arising from the *γδ*-addition of diazoacetate to the parent trimethylsorbic ester.

The residual part of the hydrolysate, from which no further crystallisation occurred, was treated with ozone, followed by hypobromite oxidation by the same procedure as above, so as to give a mixture of both *cis*-, and *trans*-caronic acids, also indicating the *γδ*-addition. The addition reaction in the present case is considered to proceed through the corresponding pyrazoline-intermediate in a way as was discussed in Part VI[3].

The geometrical configuration of α, β-ethylenic linkage of the α, β, δ-trimethylsorbic ester used is unknown, though stereochemical consideration on the atomic model favours the *trans*-configuration for this acid because there is considerable steric hindrance in the *cis*-isomer, and there has not yet been developed an experimental method for assigning geometrical configuration to the tetra-substituted ethylenic linkage.

EXPERIMENTAL

All melting points and boiling points were uncorrected. Ultra-violet light absorptions were determined in ethanol on a Beckman model DU quartz spectrophotometer. Infra-red spectra were recorded in Nujol on a Perkin Elmer double-beam spectrophotometer. Microanalyses were by the Microanalytical Division, Prof. Mitsui's Laboratory.

Methyl α, β, δ-Trimethyl-β-hydroxy-$\Delta^{\gamma\delta}$-hexenoate (I) About 50 ml. of a mixture of mesityloxide, b.p. 129–130°, 33 g. (0.3 mole.) and methyl α-bromopropionate, b.p. 48°/12 mm., 50 g. (0.3 mole.) in 50 ml. of dry benzene were added on 22 g. (0.3 atom.) of activated zinc, then it was warmed with effective stirring until a vigorous reaction set in. Addition of a bit of copper powder and/or mercuric chloride initiated the reaction smoothly. The remaining mixture was then run in at such a rate that the steady reflux of benzene was under control.

It was then warmed under reflux for an additional half an hour, cooled, poured into an ice-cold solution of dilute sulphuric acid. After removal of the unchanged zinc, the organic layer which separated, was washed with several portions of water and dried over

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[2] I. A. D'yakonov; *Zhur. Obshchei. Khim,,* **21**, 839 (1951): *C. A.*, **46**, 439 (1952).

[3] Y. Inouye and M. Ohno; *Bull. Inst. Chem. Res. Kyoto Univ.* **33**, 237 (1955): *Botyu Kagaku*, **20**, 136 (1955).

anhydrous magnesium sulphate. Replacement of zinc by magnesium, and a prolonged refluxing time increased the high boiling polymer and reduced the yield of the β-hydroxyester desired. The solvent was removed through a column and the residue was fractionated under a reduced pressure to give the methyl ester (I). b.p. 66–7°/2.5 mm., n_D^{20} 1.4540, $R_{D(obs.)}$ 51.61, $R_{D(calc.)}$ 51.12, yield 34–39 g. When ethyl α-bromopropionate was used, the corresponding ethyl ester was obtained. b.p. 77–8°/2.5 mm., n_D^{20} 1.4493, $R_{D(obs.)}$ 56.31, $R_{D(calc.)}$ 55.76, yield 36–41 g.

Methyl α, β, δ-Trimethylsorbate (II) The Reformatski ester (I) was dehydrated by refluxing with a slight excess of phosphorus pentoxide in dry benzene for an hour.

Methyl ester, b.p. 68–9°/7 mm., n_D^{20} 1.4650, $R_{D(obs.)}$ 49.52, $R_{D(calc.)}$ 49.11, yield 55–58%. *Ethyl ester,* b.p. 70–71°/5 mm., n_D^{20} 1.4610, $R_{D(obs.)}$ 54.43, $R_{D(calc.)}$ 53.76, yield 55%.

Hydrolysis of the ester (II) with ethanolic potassium hydroxide gave the free acid, b.p. 97°/4 mm., n_D^{20} 1.4809; p-*Iodophenacylester* m.p. 41–2° (*Anal.* Found: C, 51.10; H, 4.94. Calcd. for $C_{17}H_{19}O_3I$: C, 51.27; H, 4.81).

Upon hydrogenation over a platinum catalyst, the acid (1 g.) absorbed 292 ml. (at 21°) of hydrogen (equivalent to 1.88 double bond), yielding *α, β, δ-trimethylcaproic acid,* b.p. 127–8°/16 mm., n_D^{20} 1.4365; *Amide* m.p. 126–7° (*Anal.* Found: C, 68.81; H, 12.15. Calcd. for $C_9H_{19}ON$: C, 68.79; H, 12.18), *Methyl ester* b.p. 76–8°/15 mm., n_D^{20} 1.4240.

Addition of Ethyl Diazoacetate to Methyl α, β, δ-Trimethylsorbate About 1 g. of ethyl diazoacetate was added to a solution of methyl α, β, δ-trimethylsorbate (50 g., 0.3 mole.) in an equal volume of ligroin containing 1 g. of copper powder, the mixture was heated until reaction set in with the evolution of nitrogen.

The remaining diazoacetate (16 g., total 0.15 mole.) was then added at such a rate that the steady reflux and the smooth evolution of nitrogen maintained without external heating. After removal of the solvent and unreacted sorbate under a reduced pressure, the residual adduct was fractionated to give four fractions within the range of b.p. 95–115°/1.5 mm., main fraction distilling at 103–4°/1.5 mm., n_D^{20} 1.4660, $R_{D(obs.)}$ 68.56, $R_{D(calc.)}$ 68.27, yield 13 g.

These fractions were separately hydrolysed with alcoholic potassium hydroxide, and the isolated viscous acids were kept cold for a week or so. Partial crystallisation occurred, and the separate crystallisation gave an acid $C_{11}H_{16}O_4$ m.p. 164–5° (*Anal.* Found: C, 62.26; H. 7.88. Calcd. for $C_{11}H_{16}O_4$: C, 62.25; H, 7.60); *equiv.* Found 106.8. Calcd. for $C_9H_{14}(CO_2H)_2$ 106.1; λ max 238 mμ, ε, 19,700; for infrared spectrum see Fig. in the text.

Di-p-phenylphenacylester m.p.115°(*Anal.*Found: C,77.76; H, 6.01. Calcd. for $C_{39}H_{36}O_6$: C, 77.98; H, 6.04).

Ozonisation:—The acid m.p. 164–5° (1 g.) in chloroform was treated with an excess of ozonised oxygen for several hours at 0°.

The solvent was removed under a reduced pressure, and the ozonide decomposed with addition of water by warming on a water bath for ten minutes. The aqueous solution was concentrated, but no crystallisation occurred. This was then taken up with alkali, poured into a cold solution of sodium hypobromite (2.3 g. bromine in 40 ml. of 5% sodium hydroxide solution). After an hour's stirring, the excess of hypobromite was decomposed by addition of hyposulphite, acidified with dilute sulphuric acid and throughly extracted with ether. After removal of ether, the residue was recrystallised several times from water to yield *cis*-caronic acid (150 mg.) m.p. 171–2° (*Anal.* Found: C, 52.85; H, 6.01. Calcd. for $C_7H_{10}O_4$: C, 53.16; H, 6.39).

Two grams of the mother liquor, from which the partially crystallised acid was removed, was treated with ozone and then with sodium hypobromite in exactly the same way as mentioned above.

The ether extract, on evaporating, deposited a crystalline mass (ca. 0.8 g.) and to this 9 ml. of 20% aqueous ammonia was added and evaporated to dryness. The ammonium salt was ground up with cold absolute ethanol. The insoluble salt was dissolved in a small amount of water, acidified with dilute sulphuric acid and repeatedly extracted with ether, the ether extract, on evaporating, gave *trans*-caronic acid (230 mg.) which melted at 212° after several recrystallisations from water. (*Anal.* Found: C, 53.33; H, 6.20. Calcd. for $C_7H_{10}O_4$: C, 53.16; H, 6.37).

The ethanol-soluble fraction of the ammonium salt was evaporated, acidified with dilute sulphuric acid and repeatedly extracted with ether. After removal of the solvent, the residue was recrystallised from water so as to give *cis*-caronic acid (200 mg.), m.p. 173–4° (*Anal.* Found: C, 53.55; H, 6.19. Calcd. for $C_7H_{10}O_4$: C, 53.16; H, 6.37).

The authors are indebted to Prof. S. Takei for his kind advice and to the Ministry of Education for the grant.

[Bull. Agr. Chem. Soc. Japan, Vol. 20, No. 2, 80~83 pp, 1956]

The Photochemical Reaction Products of Indole in Sunlight(1)

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Received March 19, 1956

From the transformation products of indole by the action of sunlight, a crystalline material, m.p. 245° (dec.) was isolated and identified as 2, 2-diindyl-ψ-indoxyl (II), not being the ether of indoxyl proposed by B. Oddo.

The identification of anthranilic acid (III) which occurred simultaneously in the products was performed by separation using counter-current process, followed by the paper chromatographic and spectrophotometric methods.

These results indicate a possible mechanism of photochemical transformation in which both oxidative degradation and oxidative addition took place.

In their studies on the photochemical degradation products of L-tryptophan, the present authors2) showed that kynurenine was isolated in the form of crystalline sulfate with simultaneous occurrence of some indole derivatives which changed readily into indirubin and indigo. Those results suggested that tryptophan was attacked in both the indole ring and the side chain. It is now interesting to investigate the behavior of indole (I) itself when subjected to the action of sunlight. B. Oddo3) in 1916 first observed that indole was converted to a yellow powder, m.p. 207° (dec.), $C_{16}H_{12}ON_2$, on exposure to sunlight and regarded the compound as an ether of indoxyl.

Since this observation, little efforts have been made in order to identify the products or to establish the mechanism of the photochemical reaction.

The present authors have now found that a yellow crystalline material, m.p. 245° (dec.), was obtained by a procedure similar in principle to Oddo's. The chemical and physical properties corresponded closely to 2, 2-diindyl-ψ-indoxyl [2, 2-bis-(3-indyl)-indoxyl]4), which Seidel had first synthesized from indole by treatment with $NaNO_2$ in dilute sulfuric acid. Moreover, additional evidence was obtained by comparison with synthesized 2, 2-diindyl-ψ-indoxyl. When the formation of the trimer condensation product by action of sunlight is considered, it is significant that an addition and an oxidation of indole are caused by light as well as the formation of the trimer product from indole itself by treatment of peracetic acid. On the other hand, a decomposed product, anthranilic acid (III), was identified in considerable amount by means of paper chromatography and ultraviolet absorption spectrum. The occurrence of anthranilic acid accompanied the formation of larger amount of 2,2-diindyl-ψ-indoxyl. Anthranilic acid has not previously been obtained from indole by the photochemical treatment. But the finding seems to be analogous to the fact2) that kynurenine is formed from tryptophan under similar conditions. Although the course of

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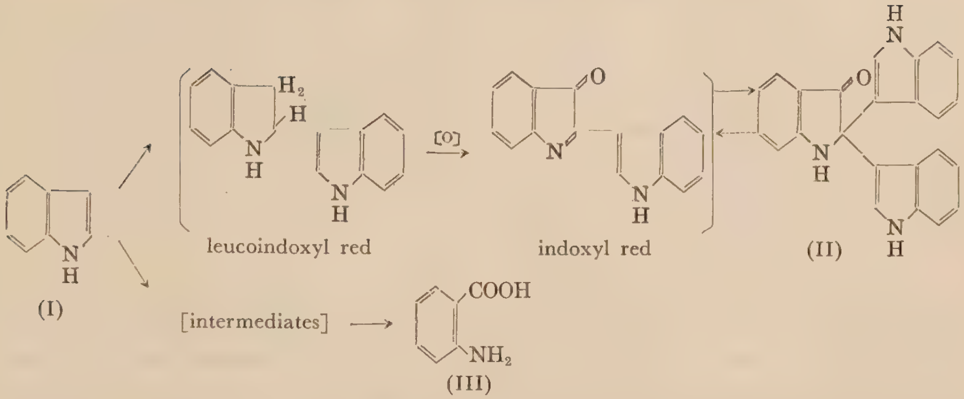
(1) 4th. comm. on "Products of Photochemical Degradation of Tryptophan." The preceding paper in this series was published in the *J. Agr. Chem. Soc. Japan*, **29**, 817 (1955). Reported at the 30th aniversary meeting of the Biochemical Society of Japan at University of Tokyo, November, 1956.

2) S. Sakamura and Y. Obata, *J. Agr. Chem. Soc. Japan*, **29**, 616 (1955).

3) B. Oddo, *Gazz. Chim. Ital.* **46**, I, 323 (1916). *C. A.* **11**, 1143 (1917).

4) P. Seidel, *Chem. Ber.* **77**, B. 794 (1944). *C. A*, **44**, 609 (1950). He referred to it as "2,2-bis-(3-indolyl)-pseudoindoxyl.



the conversion of indole to anthranilic acid was ascertained experimentally, a possible intermediate such as indoxyl may be presumed. Therefore, in addition to Witkop's postulation[5] on the mechanism in relation to oxidation, the present authors proposed a mechanism of the photochemical transformation of indole itself. It is shown in the above mentioned scheme.

EXPERIMENTAL AND RESULTS

Photochemical Treatment of Indole

Two grams of indole, m.p. 52–53°, recrystallized from petroleum ether were dissolved in 500 ml of 95% ethanol and diluted to 4000 ml with distilled water. Eight hundred ml portions of the solution each were placed in 1000 ml flasks stoppered with cotton under the condition of sterilization and exposed to sunlight for either three weeks or two months in summer. The colorless solution became red-brown and the precipitates gradually separated. In all cases flasks kept in the dark remained unchanged.

Separation and Identification of the Products

From the irradiated contents started from two grams of indole, a brown precipitate was filtered, and the filtrate reserved for use in the identification of anthranilic acid as described below in this paper. The brown precipitate was refluxed in petroleum ether to remove the remaining indole. The residue was dissolved in 95% ethanol, decolorized with a suitable amount of active carbon and then filtered to give a clear alcoholic solution. When the filtrate was concentrated to a small quantity, a mass of solid materials separated. It was collected by filtration and redissolved in ethylacetate, followed by addition of aqueous methanol and then evaporation. Such a recrystallization was repeated seven times, finally yielding 0.17 grams of yellow crystals (plates), m.p. 245° (dec.), which was light soluble in ether, alcohol, slightly in toluene, benzene, insoluble in water and petroleum ether. Admixture with authentic specimen synthesized by method of Seidel or Witkop showed no depression in melting point. The absorption spectra of the compounds were characterized by peaks of 260, 280, 405 mμ as shown in Fig. 1. The infra red spectra of the same in Nujol are also presented in Fig. 2 and corelated with each other. These results indicated quite clearly that the substance was identical with 2, 2-diindyl-ψ-indoxyl.

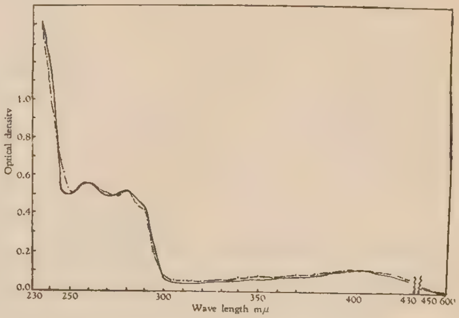


FIG. 1. Ultraviolet Absorption Spectra of Product from a Solution of Indole Irradiated in Sunlight (—·—·—) and 2, 2-Diindyl-ψ-indoxyl Synthesized by Seidel's Method (——).

Concentration of 1.4 mg/100 ml in methanol.

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[5] B. Witkop and J. B. Patrick. *J. Am. Chem. Soc.* **73**, 713 (1951). Also refer Mustafa, *Chem. Rev.* **51**, 1 (1952). Remo De Fazi, *Gazz. Chim. Ital.* **54**, 85 (1924). *C. A.* **18**, 2173 (1924). O. Schmitz-Dumont, K. Hamann and K. H. Geller, *Ann.* **504**, 1 (1933).

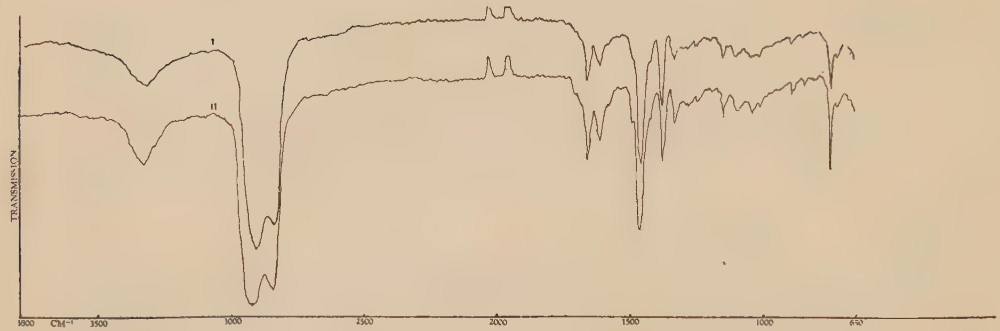

FIG. 2. Infra Red Spectra of the Product (I) and Authentic 2, 2-Diindyl-ψ-indoxyl (II) in Nujol.

Anal. Found: C, 78.66; H, 4.91; N, 11.50; Molecular weight (RAST method) 382, 397, Calcd. for $C_{24}H_{17}ON_3$: M.W. 363; C, 79.32; H, 4.72; N, 11.57%. Moreover, a solution of the compound instantly became red upon the addition of concentrated hydrochloric acid, 50% sulfuric acid or cold Millon's reagent. But upon the addition of acetic acid or formic acid also it gradually assumed a red coloration. The change of color originating from the compound is a general property of an acidic solution and is only accelerated by addition of $Hg(NO_3)_2$ and $HgSO_4$. When a suspension of 50 mg of the compound in 20% hydrochloric acid was refluxed for 5 hours, the reaction mixture turned pink, subsequently purple and finally resulted in bluish green precipitates. These precipitates were soluble in chloroform and methanol. The absorption spectrum in methanol was estimated and compared with authentic indirubin (see Fig. 3). The material did not appear to be identical to indirubin.

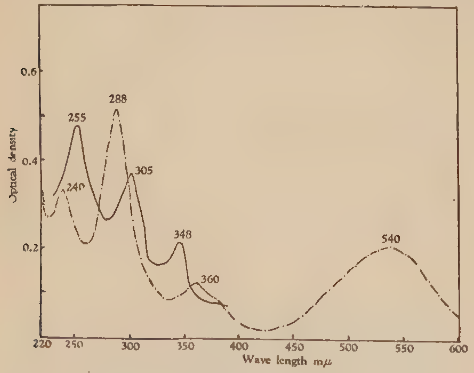

FIG. 3. The Spectrophotometrical Comparison with the Substance Formed from 2, 2-Diindyl-ψ-indoxyl by Treatment with Hydrochloric Acid (—·—·—) and Authentic Indirubin (——).

Dissolved in methanol.

Synthesis of 2, 2-Diindyl-ψ-indoxyl (*1*) *By the Method of Seidel*[1] According to the procedure described by Seidel, two grams of pulverized indole were treated with 0.4 g of sodium nitrate and 18 ml of concentrated sulfuric acid after which purification was achieved as described above. The final yield in pure state was 0.705 g, it melted at 245° (dec.), no depression was shown on mixing with pure sample of the photochemical product.

(*2*) *By the treatment of peracetic acid*[5] One and five-tenths gram of indole was treated with glacial acetic acid and 1.5 ml of hydrogen peroxide (30%) by the procedure described by Witkop. The resulting products were somewhat different from those of Witkop; one of these coincided with the higher melting isomer (2, 2-diindyl-ψ-indoxyl) and another was indigo blue. The former melted at 244–5° (dec.), weighing 174 mg; the latter sublimed unchanged at approximately 285° and did not decompose at 330° in sealed tube, weighing 95.2 mg and the absorption spectrum was characteristic for indigo blue (Fig. 4).

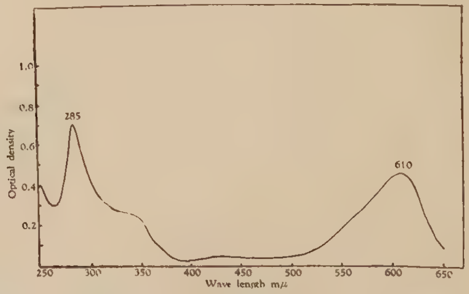

FIG. 4. The Absorption Spectrnm of Indigo Produced from Indole by Peracetic Acid Oxidation.

Concentration of 1.1 mg/100 ml in chloroform.

Identification of Anthranilic Acid *Paper chromatography* After removal of the precipitates (2, 2-diindyl-ψ-indoxyl) from the irradiated solution (corresponding to 5.0 g indole) as described above, the resultant filtrate was concentrated under reduced pressure, and filtered to yield a clear solution. Then a part of the solution was subjected to paper chromatographic examination. As shown in Table I, a positive spot for Ehrich's aldehyde reaction, diazotization reaction (using N-(1-naphtyl)-ethylene-diamine for coupling reagent) and fluorescence under the ultraviolet irradiation, were quite similar to corresponding properties of anthranilic acid. When chromatographed in the solvent (water-saturated butanol), another spot (R_F 0.34) behaved like that of anthranilic acid except for delay of the development for both color reactions. No further detailed examination for the identification of the material was made.

TABLE I
R_F VALUES OF SOME PRODUCTS AND ANTHRANILIC ACID

	Product			Authentic anthranilic acid
	Ehrich's aldehyde reaction	Diazotization reaction	Fluorescence	
(1)	0.64 0.34	0.65 0.34	0.65	0.64
(2)	0.79	0.79	0.79	0.80
(3)	0.89	0.90	0.90	0.90

(1) water-saturated butanol
(2) methanol-butanol benzene-water (4. 2. 2. 2.)
(3) butanol-acetic acid-water (4. 1. 5.)

Ultraviolet absorption In order to purify the material corresponding to anthranilic acid, the remaining solution was separated by counter-current distribution method. The procedure was carried through by using 50 tubes, ethylacetate-N/10 phosphate buffer (pH 7.4) as solvent. The upper layers of No. 45 and 46 tube which had been stronger for the fluorescence and the color reactions, were combined together, dried with anhydrous sodium sulfate, evaporated to dryness in a vacuum desiccator. One part of the residue was directly dissolved in N/10 phosphate buffer (pH 7.4) and the other, after the paper chromatographic separation, was submitted to elution of the spot corresponding to anthranilic acid with N/10 phosphate buffer. Then the absorption spectra of the two materials were measured. They are shown in Fig. 5. The absorption maximum at 309 to 310 mμ coincided exactly with that of authentic anthranilic acid.

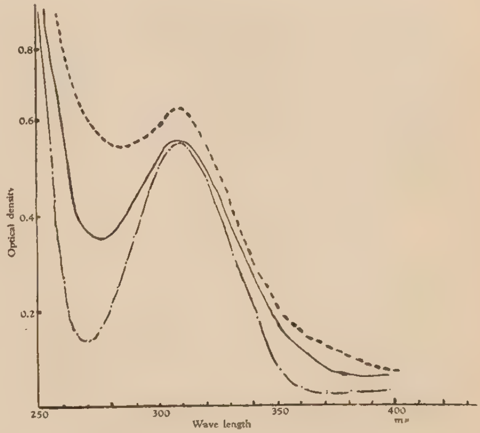

FIG. 5. Ultraviolet Absorption Spectra of Anthranilic Acid and of the Product from a Solution of Indole Irradiated in Sunlight.

All samples were measured in N/10 phosphate buffer (pH 7.4). product separated by counter-current distribution method (----), eluted substance from the paper chromatogram with the buffer after separation by counter-current distribution method (———), authentic anthranilic acid (—•—•—•).

The authors are indebted to the Central Research Institute of the Japan Monopoly Corporation for infra red data and also to the Laboratory of Microanalysis, University of Tokyo, for the analytical results. The cost of the work was partly defrayed from a Grant in Aid for Scientific Research from the Ministry of Education.

SUMMARY

The separation and identification of the products transformed from indole itself by the action of sunlight were studied. The following results were obtained.

(1) Exposure to sunlight resulted in the decomposition of indole accompanied with brown precipitates, but in case of dark experiment no change was observed.

(2) A crystalline material of yellow plates, m.p. 245°, was obtained, it was identical to 2, 2-diindyl-ψ-indoxyl.

(3) Among other products, anthranilic acid was identified by paper chromatography, ultraviolet spectra or counter-current distribution procedure.

(4) Therefore, a possible mechanism for the transformation of indole was proposed as shown in the scheme.

[Bull. Agr. Chem. Soc. Japan, Vol. 20, No. 2, 84~88 pp, 1956]

Studies of the Essential Oil of *Mentha rotundifolia*

Part I. Isolation of Rotundifolone, a New Terpenic Ketone.*

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Received March 22, 1956

Rotundifolone, a new terpenic ketone, $C_{10}H_{14}O_2$ has been isolated from the essential oil of *M. rotundifolia* in Japan. This ketone is highly dextro-rotatory, and has a low melting point 27.5°. The wave lengths of the ultraviolet absorption maxima of rotundifolone and its semicarbazone are 260 mμ ($\log \varepsilon = 3.95$) and 273 mμ ($\log \varepsilon = 4.12$) respectively. This indicates that rotundifolone has an α,β-unsaturated carbonylic system.

Concerning the essential oil of *Mentha rotundifolia* cultivated in Japan, physical properties of the oil were reported[1] but not its chemical constituents. In Europe there were many chemical investigations[2][3] and recently A. F. Costa[4] (1945) found limonene, pinene and esters in the essential oil of *M. rotundifolia* produced in Portugal.

J. Sfiras[5] (1952) reported that Timijamint oil (from *M. rotundifolia*) contained approximately 0.4% isopulegone as well as 0.5% borneol free or esterified. R. Hegnauer[6] (1953) found carvone in the oil of Netherlands.

In the previous investigation[7] the author showed the half wave potentials of various ketones which were contained in the peppermint oil, and suggested the use of polarographic method for examining the quality of the oil. It was pointed out by this method that the essential oil of *M. rotundifolia* cultivated in Japan contained a substance which was reduced at the dropping mercury cathode extremely easier than any other essential oil of *Mentha* plants. These results were confirmed by the experiments as regards the oil produced in Japan in 1954 and 1955. (Fig. 1)

The ultraviolet spectrum of this oil had a maximum absorption at the wave length 260 mμ without exception. When this oil was reacted with 20% sodium sulfite under stirring on the boiling waterbath, a greater part of the oil went into solution. The ultraviolet spectrum of the unreacted residual oil had low absorption intensity at the wavelength 260 mμ. This indicates that the substance having a maximum absorption at 260 mμ, reacted with the sodium sulfite solution. Consequently it seemed that the principal component of the oil of Japanese *M. rotundifolia* would be a kind of $\alpha.\beta$-unsaturated ketone.

But this ketone could not be regenerated from its bisulfite addition product with sodium hydroxide solution by the usual method. The optical rotation of the essential oil was highly dextrorotatory. Considering the fact above mentioned, it seems that Japanese oil is quite different from the European oil.

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* This report was presented at the annual meeting of Agric, Chem. Soc. Japan, Univ. of Tokyo, March, 30, (1955)

[1] T. Nagasawa: *Okayama Pref. Agric. Exp. Station, Special Bulletin*, No. **50**. (1954).

[2] *Berichte von Schimmel:* April, 80. (1907).

[3] R. Rovesti: *Profumi Italici.*, **3**, 178. (1925).

[4] A. F. Costa: *Bol. escola, farm, Univ. Coinbra* (Portugal) **5**, 90. (1945).

[5] J. Sfiras: *Researches* **22**, (1952), C. A. **46**, 6333 (1952).

[6] R. Hegnauer: *Ber. Schweiz botan. ges.* **63**, 90 (1953), C. A. **48**. 11733. (1954).

[7] S. Shimizu *Journal of the Shinshu Univ.* **4**, 313 (1954).

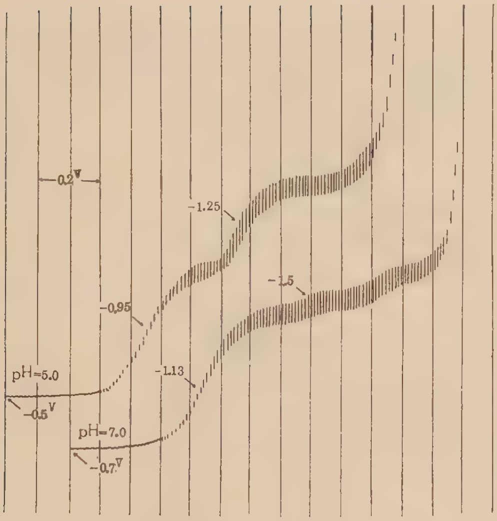

FIG. 1. Polarograms of the Essential Oil of Japanese *M. rotundifolia.*

S=1/20; oil wt. 2.13 mg, MeOH 3ml. Sörensen buffer sol. 6ml. 1N-LiCl=1ml., galvanometer senc. 5.23×10^{-9}A/mm/m, m=1.57mg/sec. t=4.5sec/drop.

It happens often that the same morphological species contains different components, when cultivated at different localities. For instance, is it well known that *Mentha viridis* oil of Japan and Europe contains chiefly carvone, but linalool is found in the Russian oil as a principal component. The author's oil was compared with two of the authentic oils of Japan Agricultural Experiment Station for peppermint (Okayama and Hokkaido). For optical rotation and ultraviolet spectra, no significant difference was recognized among them, as shown in Table I.

Consequently, it is clear that *M. rotundifolia* cultivated in Japan belongs to the same series, but differs from the European one without doubt. The Japanese oil has a high specific gravity and a high refractive ratio than the European oil.

The substance having λ_{max} 260 mμ in Japanese oil could not be isolated by the usual bisulfite method, and the chromatographic separation using alumina or silica absorbent was also without success. By vacuum fractional distillation four distillates were obtained, and the ultraviolet absorption of each distillate was measured. The distillate 95°/3–4 mm was found to have the strongest absorption at the wave length 260 mμ. (Fig. 2) By cooling this fraction with a freezing mixture, a ketonic substance was obtained as a solid. By recrystallization from cold petroleum ether (b.p. 30–40°), the low melting, colourless substance was obtained and melted at 27.5°. The crystal shows strong absorption in ultraviolet region, λ_{max} 260 mμ ($\log \varepsilon=3.95$ in ethanol) (Fig. 3)

This substance gives easily a monosemicarbazone in pyridine methanol which melts at 180° and has strong absorption at the wave length 273 mμ ($\log \varepsilon=4.12$, in ethanol) (Fig. 3)

TABLE I

PHYSICAL PROPERTIES OF THE ESSENTIAL OIL OF JAPANESE *M. rotundifolia.**

Place.	Year of harvesting	$[\alpha]_D^{15}$	Maximum of wave length.	Absorbancy at 260 mμ (10^{α}/ml)	n_D^{25}
Ina.	(1953) Oct.	+101.2°	260 mμ	0.461	1.5051
〃	(1954) Oct.	+ 85.4	〃	0.450	1.5052
〃	(1955) July.	+155.5	〃	0.470	1.5052
〃	(〃) Oct.	+125.0	〃	0.492	1.5053
Hokkaido.	(〃) Oct.	+ 86.3	〃	0.490	1.5052
Okayama.	(〃) Nov.	+ 82.3	〃	0.260	1.5000

* Properties of *M. rotundifolia* oil in Europe were as follows.

Place.	Year of harvesting.	Optical rotation.	Ref. ratio.	Investigator.
Itally.	(1925)	+19. 3	1.4881	Rovesti3)
〃	(1907)	−37.30	1.49471	Schimmel2)
Portugal Coja,	(1942)	−23.52	1.4757	Costa4)
Portugal Preixo.	(1942)	− 0.22	1.4840	〃

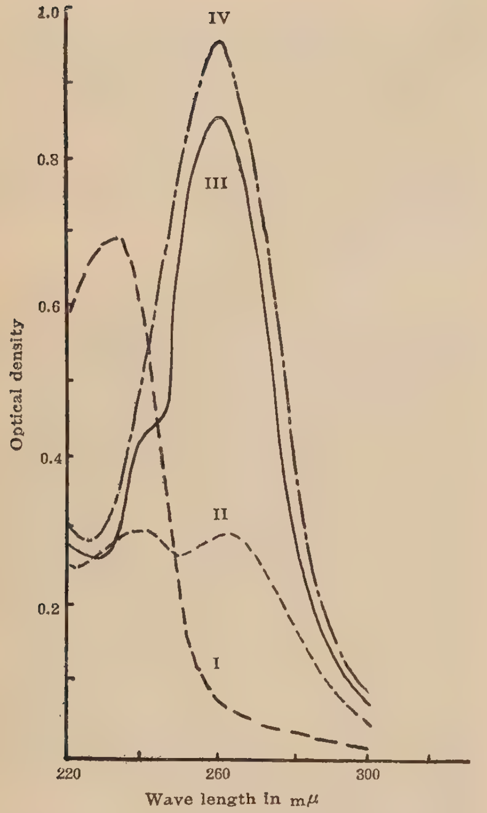

FIG. 2. Ultraviolet Absorption Spectra of Fractional Distillates of *M. rotundifolia* Oil Cultivated in Hokkaido.

conc. of oil, (γ/ml) I=13.8, II=21.8, III=19.6, IV=18.8, in methanol.

From the results of elementary analysis of original ketone and its semicarbazone, the new terpenic ketone is shown to have the molecular formula $C_{10}H_{14}O_2$. The author named this ketone as "rotundifolone".

Rotundifolone does not give ferric chloride reaction in methanol nor in aqueous suspension, but shows positive Malaprade reaction. It reduces ammoniacal silver nitrate and Fehling solution.

Physical properties are as follows;

$[\alpha]_D^{10}+199.6°$, n_D^{30} 1.5045, d_4^{30} 1.053.

Rotundifolone is soluble in ethanol, methanol, benzene and petroleum ether but not in water and aqueous alkali. The oil

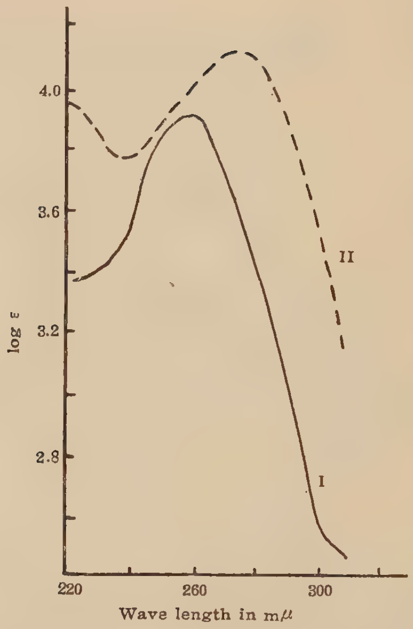

FIG. 3. Ultraviolet Absorption Spectra of Rotundifolone I and Semicarbazone II in Ethanol.

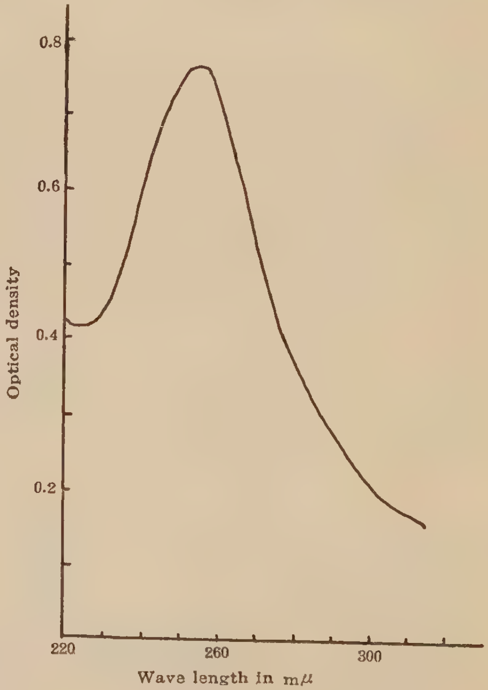

FIG. 4. Ultraviolet Absorption Spectrum of the Oil Regenerated from Rotundifolone Semicarbazone:
conc. of oil 23.7γ/ml. in methanol.

which is regenerated from the rotundifolone semicarbazone by oxalic acid, has a strong absorption in ultraviolet region, λ_{max} 255 mμ (Fig. 4) and shows positive ferric chloride reaction. Calculated from the ultraviolet absorbancy of the fractional distillates and the yield of the semicarbazone of the original oil, the essential oil of Japanese *Mentha rotundifolia* seems to contain about 50% rotundifolone.

EXPERIMENTAL

Cultivation of *Mentha rotundifolia* The root cuttings in this investigation were taken from the Konosu Agricultural Experiment Station in 1951. After harvesting, the rootstocks were preserved in soil for the propagation purpose of next year. The method of cultivation was almost the same as the usual one for Japanese peppermint. The planting of root cuttings was carried out in late April. Manurial treatments were as follows; N, 2.0 kg. P, 1.2 kg. K, 1.3 kg. per 1.0 *are* including farmyard-manure 20 kg. The plants were harvested twice a year, in July and in October.

Preparation of the Essential Oil After crops were dried in shade for 5–7 days, about 1 kg. of cuttered herbs and 3 l of water were placed in a distillation kettle fitted with water condenser and distilled for 2–3 hours to get 1.5–2 l of distillate. Most of the oil was floating on the surface of condensate, but a minor part settled at the bottom. The distillate was saturated with sodium chloride and extracted with ether. The ether extract was dried over anhydrous sodium sulphate. Removal of the ether left the essential oil.

TABLE II

THE YIELD OF THE ESSENTIAL OIL

Date of harvesting	Wt. of fresh herbs	Wt. of oil	Yield
1953, July, 27.	2100 g	1.3 g	0.06%
Oct. 15.	1000	1.2	0.12
1954, July, 13.	5600	6.2	0.11
Oct. 10.	6600	8.4	0.13
1955, July, 13.	14300	26.8	0.19
Oct. 7.	17200	22.6	0.13

Polarography To 3 ml. of the methanol solution which contained 2.13 mg. of the essential oil of *Mentha rotundifolia*, 1 ml. of N-LiCl solution and 6 ml. of Sörensen buffer solution were added and shaken. This was put in an electrolytic cell from which oxygen was driven out by hydrogen gas. The polarograms were taken at 25° ±0.30 C. (Fig. 1)

$\pi\frac{1}{2}$ $-0.92^{\text{Volt, S.C.E.}}$ for pH=5.0; $\pi\frac{1}{2}$ $-1.13^{\text{Volt, S.C.E.}}$ for pH=7.0.

The small wave ($\pi\frac{1}{2}$ −1.51 Volt, pH=7.0) seemed to show the existence of carvone.

Ultraviolet Absorption Spectra of the Essential Oil The ultraviolet absorption was measured in methanol or in ethanol by using a Beckman D. U. quarz spectrophotometer.

TABLE III

ULTRAVIOLET ABSORPTION OF JAPANESE OILS

Place	Wt. of oil.	λ_{max}	Optical density.	Optical density for 10γ/ml.
oil of Ina (1955)	176.5γ	260mμ	0.876	0.497
〃 Hokkaido. (1955)	110.0	〃	0.538	0.489
〃 Okayama. (1955)	452.0	〃	1.168	0.259

These results show Okayama oil contained a smaller amount of rotundifolone compared with other oils.

Isolation of Rotundifolone by Vacuum Fractional Distillation Four fractions were separated by using a small Claisen flask fitted with a short column. (2 cm. of length).

TABLE IV

DISTILLATION OF HOKKAIDO OIL (TOTAL WEIGHT 10.0 g.)

Fraction	Bath temp.	Pressure	Distil temp.	Yield	λ_{max}
I	100°	6mm	50–85°	0.5g	231mμ
II	150	8	86–105	1.2	240,260
III	150	10	106–118	4.5	260
IV	150	10	119–121	1.2	260

Fraction I oil is levorotatory $[\alpha]_D^{10}$ −23°, but further investigation could not be continued for its small amount. (Fig. 2)

TABLE V

DISTILLATION OF INA OIL (TOTAL WEIGHT 14.4 g.)

Fraction	Bath temp.	Pressure	Distil temp.	Yield	λ_{max}
I	100°	3–4mm	40–60°	0.7g.	228mμ
II	118–120	〃	95	11.1	260
III	130–150	〃	95–105	1.05	260
IV	150–170	〃	103–136	0.4	260
Residue.				1.0	

When the main distillate fraction II was cooled to −10° in a freezing mixture, the oil solidified (m.p. 17–19°), and by repeated recrystallization from cold petroleum ether, it gave the colourless needles of ultimate m.p. 27.5°. *Anal.* Found: C, 72.8, 72.9; H, 8.28, 8.45; Calcd, for $C_{10}H_{14}O_2$: C, 72.28; H, 8.45% λ_{max} 260mμ, (log ε=3.95, in ethanol) $[\alpha]^{10}_{D}$+199.6°, (c=2 in methanol) n^{30}_{D} 1.5045, d^{30}_{4} 1.053.

Semicarbazone of Rotundifolone To 40 ml. of methanol containing 5 g. of the essential oil, 5 ml. of pyridine and the 5 ml. aqueous solution of 2.5 g. of semicarbazide hydrochloride were added. The solution was allowed to stand overnight and then filtered. Yield, 3.1 g. After recrystallization from methanol, the semicarbazone melted at 180°. Ultra violet spectrum; λ_{max} 273mμ. (log ε=4.12 in ethanol.) *Anal.* Found: C, 59.03, 58.85; H, 7.52, 7.45; N, 18.78, 18.54. Calcd. for $C_{11}H_{17}O_2N_3$: C, 59.17; H, 7.68; N, 18.82%.

The Oil Regenerated from Rotundifolone Semicarbazone Semicarbazone (2.35 g.) and oxalic acid (0.685g.) were poured into 50 ml. of water and boiled for thirty minutes gently. After cooling, the solution was extracted with ether for ten hours. Ether solution was washed with the saturated sodium bicarbonate solution, and dried over anhydrous sodium sulfate. Removal of the ether left 1.1g. of the oil which gave postitive ferric chloride reaction and had strong absorption in the ultraviolet region, λ_{max} 255 mμ in methanol. (Fig. 4) This indicates that the isomerization occurred in the course of regeneration in acid media. The detail of the reaction will be reported later.

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[Bull. Agr. Chem. Soc. Japan, Vol. 20, No. 2, 89~94 pp, 1956]

Studies on Chrysanthemic Acid

Part III. Oxidation and Reduction Products of Chrysanthemic Acid

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Starting from (±)-*cis*- and (±)-*trans*-chrysanthemic acid several oxidation and reduction products were obtained: These are shown in the figure.

Chrysanthemic acid (I) is a component of pyrethrin I and cinerin I which are insecticides occurred in Pyrethrum flowers (*Chrysanthemum cinerariaefolium*). The naturally occurring chrysanthemic acid is *d-trans* isomer. Synthetic chrysanthemic acid contained in commercial allethrin is a mixture of *dl-cis* and *-trans* isomers. The authors attempted oxidation and reduction of these acids and obtained several compounds which hitherto had been unknown.

R. Yamamoto[1], oxidized *d-trans*-chrysanthemic acid with permanganate and obtained dihydroxychrysanthemic acid, m.p. 145°. The authors applied the reaction to *dl-trans* and *dl-cis* chrysanthemic acids and obtained three dihydroxy acids which melted at 144°, 180° and 240°. The first two were from *trans*-acid and the third from *cis*-acid.

Chrysanthemols (V) were obtained by lithium aluminum hydride reduction of ester or acid chloride of *dl-cis* and *dl-trans* chrysanthemic acids[2]. The compounds have a terpene alcohol like fragrance, and seemed to be unstable to acid[1]. By ozonolysis of the compounds, aceton and oily hydroxylic acids (VI) were obtained, the former was identified as 2,4-dinitrophenylhydrazone and the latter acids were derived to *cis*- and *trans*-caronic acids by oxidation with potassium permanganate. *cis*-Chrysanthemol was also oxidized with potassium permanganate and the authors obtained oxalic acid, m.p. 100°, and to the authors' surprise 2,2-dimethyl succinic acid, mp. 136°, also an acid which melted at 175° but could be distinguished from *dl-cis*-caronic (m.p. 174°), *dl*-terebic (m.p. 174–5°) and succinic acid by admixture. *cis*-Chrysanthemol was brominated with phosphorous tribromide and pyridine and chrysanthemyl bromide was obtained.

The problem of what kind of acids could be obtained by catalytic reduction of chrysanthemic acid is interesting in relation to the determination of pyrethrin and allethrin by the methods of catalytic reduction and of polarography. The authors reduced *cis*-chrysanthemic acid in the presence of Raney-nickel, platinum oxide and palladium oxide catalysts. At the first step of the reduction consuming one molecule of hydrogen, dihydrochrysanthemic acid (VI), m.p. 54° was obtained with each catalyst, by using Raney-nickel catalyst under pressure, a hydrocompound, m.p. 85°, probably VII was obtained which was oxidized with potassium permanganate at room temperature to a compound, m.p. 55°, the elementary analysis of which showed $C_{10}H_{18}O$. The compound is a lactone, different from dihydrochrysanthemolactone[3]

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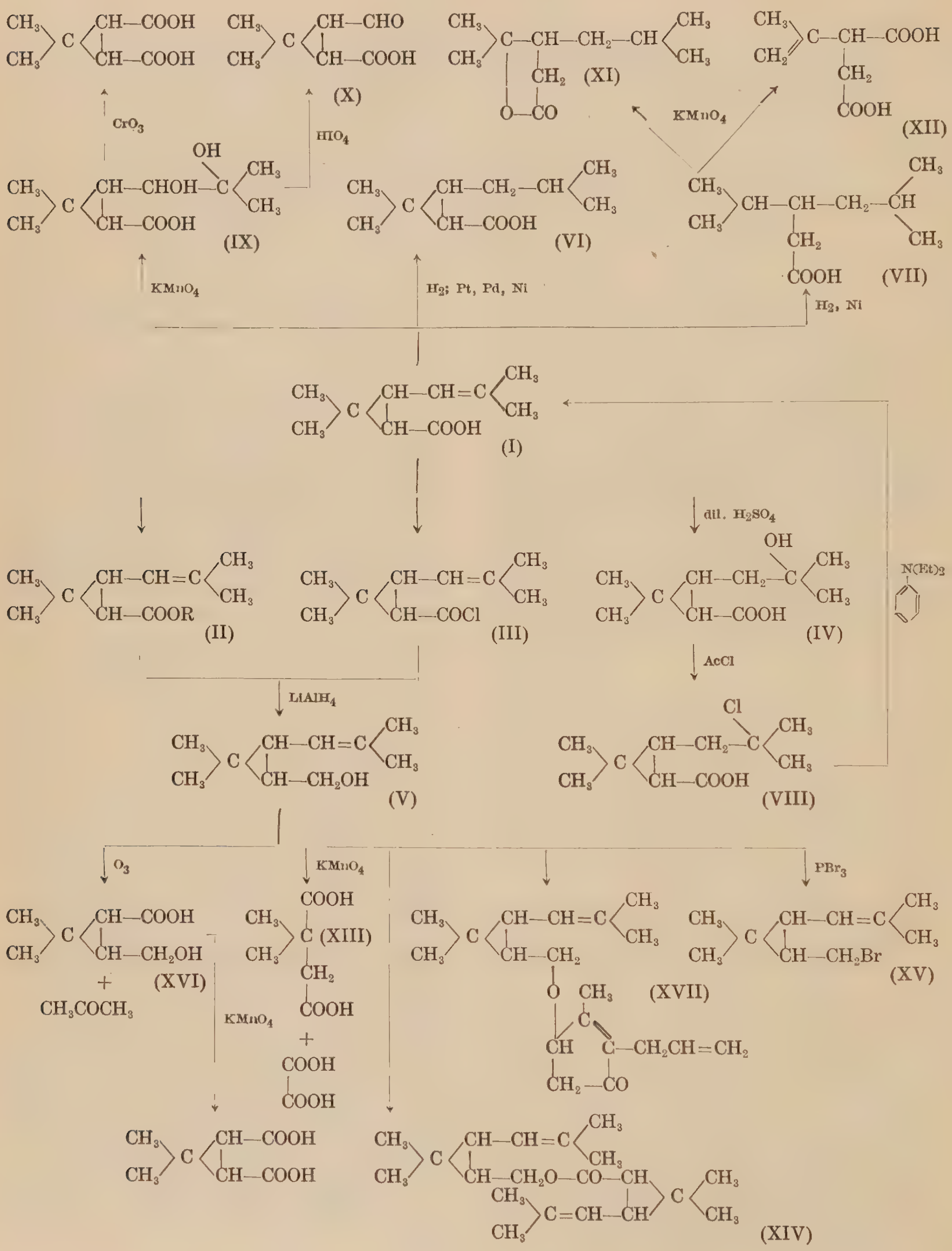
[1] R, Yamamoto, *J. Chem. Soc. Japan*, **44**, 323 (1923).

[2] Cf. Y. Inouye and M. Ohno. This Bulletin **20**, 25 (1956).

* Laboratory of Sumitomo, Chem. Co., Ltd. Osaka.

[3] L, Crombie, S.H. Harper and R.A. Thompson, *J. Sci. Food Agric.*, **9**, 421 (1951); S.H. Harper and R.A. Thompson, *ibid.*, **5**, 230 (1952).


(X)
(XI)
(XII)
CrO3
HIO4
KMnO4
(IX)
(VI)
(VII)
KMnO4
H2; Pt, Pd, Ni
H2, Ni
(I)
dil. H2SO4
(II)
(III)
(IV)
N(Et)2
AcCl
LiAlH4
(V)
(VIII)
O3
KMnO4
PBr3
(XVI)
CH3COCH3
(XIII)
(XVII)
(XV)
KMnO4
(XIV)

or pyrocin[4] on mixed melting point determination. It probably has the structure XI i.e. dihydropyrocin.

In relation to the experiments above, *dl-cis*-chrysanthemyl *dl-cis*-chrysanthemate(XIV) and *dl-cis-* and *trans*-chrysanthemyl allylrethronyl ether (XVII) were prepared, the two compounds having little insecticidal activity to the house fly.

(±)-Dihydro-hydroxychrysanthemic acid was converted to (±)-dihydro-δ-chlorochrysanthemic acid, m.p. 91–2°. The chloro acid was treated with diethylaniline and pure (±)-*trans*-chrysanthemic acid was prepared. A more easy preparation of pure (±)-*trans*-chrysanthemic acid has already been reported[5].

EXPERIMENTAL

1). Oxidation of (±)-*cis*-Chrysanthemic Acid with Potassium Permanganate. (±)-*cis*-Chrysanthemic acid (35 g) was dissolved in 500 ml of 1.6% sodium hydroxide solution and to this 21 g of powdered potassium permanganate was added in small portions under stirring and slightly warming. After diminishing the colour of permanganate, manganese dioxide was removed by filtration. The solution was acidified with hydrochloric acid and crystalline (±)-*cis*-chrysanthemic acid which separated was removed. The mother liquor was neutralized and evaporated, acidified with hydrochloric acid and extracted with ether. A portion of the extract (30 g) was crystallized and washed with ethyl acetate. The crystals (5 g) melted at 243° after recrystallization from alcohol-ethyl acetate. (*Anal.* C, 60.64; H, 8.03. $C_{10}H_{16}O_4$ requires: C, 59.5; H, 8.9%). This is an isomer of dihydroxydihydrochrysanthemic acid (X) which was oxidized to *cis*-caronic acid (m.p. 175°) with CrO_3 in acetic acid though the yield was poor.

The mother liquor was distilled and a great part of the distillate was collected at 120–137°/0.3 mm. n_D^{27} 1.4696–1.4739. Residual crystal mass was washed with ethyl acetate and less pure (±)-*cis*-dihydroxychrysanthemic acid (2.5 g) m.p. 222° was obtained. The middle fraction of the distillate was analyzed (C, 60.52; H, 8.70. Calcd. for $C_{10}H_{18}O_4$: C, 59.5; H, 8.9%). The dislillate is probably a mixture of two isomers of (±)-*cis*-dihydroxydihydrochrysanthemic acid.

In another experiment where 11 g of (±)-*cis*-chrysanthemic acid and 11 g of potassium permanganate were used, 2 g of crystalline dihydroxychrysanthemic acid was obtained.

2). Oxidation of (±)-*trans*-Chrysanthemic Acid with Potassium Permanganate. (±)-*trans*-Chrysanthemic acid (10 g) was dissolved in 20 ml of 20% sodium hydroxide solution and 400 ml of water was added. Potassium permanganate (15 g) was added in small portions to this solution under stirring and cooling in an ice bath (1 hr.) stirring being continued in a boiling water bath until the colour of permanganate disappeared. Manganese dioxide was removed and the filtrate was evaporated to ca. 100 ml. After acidifing with HCl, the solution was extracted with ether in a percolator (16 hrs.). After evaporation of solvent, the residue was left standing to crystallize. The crystals, m.p. 118–178°, were collected and recrystallized several times from alcohol and water, needles, isomer of pure (±)-*trans*-dihydroxydihydrochrysanthemic acid (IX) (2 g), m.p. 143–144°, was obtained, (*Anal.* C, 59.7; H, 8.61. Calcd. for $C_{10}H_{18}O_4$ C, 59.5; H, 8.9%). The melting point of active dihydroxydihydrochrysanthemic acid which was derived from natural (±)-*trans*-chrysanthemic acid was 146°[1]. The (±)-*trans*-dihydroxydihydrochrysanthemic acid was oxidized with CrO_3 to *trans*-caronic acid, m.p. 212°, though the yield was not so good.

Mother liquor (42 g) of the acid melting 146° (starting from 60 g of (±)-*trans*-chrysanthemic acid) was oxidized with 28 g of chromium trioxide in 150 ml of acetic acid at 15°. After evaporation of acetic acid in vacuo, water was added and the mixture was extracted with ether. (±)-*trans*-Caronic acid (6 g) was obtained from the ether extract, after evaporation of the solvent. The aqueous layer of ether extract was further extracted with ether in a percolator for 72 hrs., then ether was evaporated. Colorless crystalline acid thus obtained was recrystallized from ethanol m.p. 180°, yield 3.5 g (*Anal.* C, 59.94; H, 8.05. Calcd. for $C_{10}H_{18}O_4$: C, 59.94; H, 8.9%).

The acid was another isomer of (±)-*trans*-dihydroxydihydrochrysanthemic acid (IX) which was proved by the next experiment. The dihydroxy acid (1.0 g) was oxidized with periodic acid (from 1.2 g of potassium periodate and 0.25 ml of 95% sulfuric acid in 60 ml water) at 30° for 48 hrs. Ether extraction afforded (±)-*trans*-caronic halfaldehydic acid m.p. 80–

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[4] M. Matsui, *Botyu-Kagaku*, **15**, 1 (1950); M. Matsui, T. Ohno, S. Kitamura and M. Toyao, *Bull. Chem. Soc. Japan*, **25**, 210 (1952). S.H. Harper and R.A. Thompson, *J. Sci. Food Agric.*, **5**, 230 (1952).

[5] M. Matsui and M. Miyano, This Bulletin **19**, 159 (1955).

81° (*Anal.* C, 58.42; H, 6.50. Calcd. for $C_7H_{10}O_3$: C, 59.1; H, 7.0%). 2, 4-Dinitrophenylhydrazone was prepared and recrystallized from ethanol-ethyl acetate, m.p. 208–9° (*Anal.* C, 48.2; H, 4.0; N, 17.6. Calcd. for $C_{13}H_{14}O_6N_4$: C, 49.1; H, 3.2; N, 17.6%).

3). (±)-*cis*-Chrysanthemol, (±)-*cis*-3-Isobutenyl-2, 2-Dimethylcyclopropyl-1-Carbinol (V). (±)-*cis*-Chrysanthemoyl chloride (III) (89 g) was added to the suspension of 10 g of lithium aluminum hydride in 400 ml of dry ether at such a rate that ether refluxed gently (1.5 hr.) and stirring was continued further for 4 hrs. The reaction mixture was decomposed by adding ice water and ethereal layer was washed with 10% potassium hydroxide solution and dried over anhydrous potassium carbonate. After evaporation of the solvennt, the residue was fractionally distilled. The alcohol was collected at 108–110°/30 mm. 35 g (yield 47.5%), n_D^{22} 1.4695, colourless fragrant liquid. 3, 5-Dinitrobenzoate was prepared and recrystallized from petroleum ether, m.p. 88° (*Anal.* C, 58.6; H, 5.41; N, 8.22. Calcd. for $C_{17}H_{20}O_6N_2$: C, 58.61; H, 5.78; N, 8.05%).

Ethyl (±)-*cis*-chrysanthemate (15 g) was reduced with 1.7 g of lithium aluminum hydride in 50 ml of ether at 20°. Excess lithium aluminum hydride was decomposed with ethyl acetate and water was added. Ether layer was separated and after ether was recovered, the residue was refluxed with 40 ml of methanolic sodium hydroxide for 2 hrs; unsaponified matter was extracted with ether and the ether extract washed with water. Distillation afforded 10 g of (±)-chrysanthemol boiling at 91–2°/8 mm., (n_D^{25} 1.4699, yield 85%), 3, 5-dinitrobenzoate was identical with that derived from alcohol which was prepared from the acid chloride.

Ozonization of (±)-cis-*chrysanthemol.* Ozonized air was passed through 3 g of (±)-*cis*-chrysanthemol in 100 ml of chloroform for three hrs. After evaporation of chloroform at room temperature, ice water was added to viscous residue and the mixture was gradually heated in a water bath. The low boiling distillate was collected in the solution of *p*-nitrophenylhydrazone in 50% acetic acid. Yellow precipitates were collected and recrystallized from dilute alcohol and *p*-nitrophenylhydrazone m.p. 146° was obtained. Mixed melting point determination showed that it was acetone *p*-nitrophenylhydrazone, m.p. 146. The decomposed water solution, after neutralization with sodium bicarbonate, was steam distilled and volatile materials were removed. The solution was acidified with sulfuric acid and extracted with ether in a percolater for 30 hrs. From the ether extract an oily acid (ca. 2 g) was obtained. One gram of the oily acid was dissolved in sodium hydroxide solution and oxidized with potassium permanganate (ca. 1 g) at room temperature. Excess permanganate and manganese dioxide was decomposed with sulfur dioxide and the solution was continuously extracted with ether and oxalic acid, m.p. 100° and (±)-caronic acid, m.p. and mixed m.p. 175°, were obtained.

4). (±)-*trans*-Chrysanthemol (V). One hundred and thirty gram of methyl (±)-*trans*-chrysanthemate (b.p. 105–8°/22 mm. n_D^{22} 1.4594) was reduced by using 14 g of lithium aluminum hydride and 400 ml of ether in the same manner as that is described in the reduction of (±)-*cis*-chrysanthemic acid. Thus (±)-*trans*-chrysanthemol (95 g), b.p. 110–111°/28 mm., $n_D^{23.5}$ 1.4670, was obtained (yield 86%). 3, 5-Dintrobenzoate was prepared and recrystallized from petroleum ether m.p. 105–6° (*Anal.* C, 58.14; H, 6.01; N, 7.80. Calcd. for $C_{17}H_{20}O_6N_2$: C, 58.61; H, 5.78; N, 8.06%).

Ozonolysis of (±)-trans-*chrysanthemol.* The result was almost identical with (±)-*cis*-chrysanthemol except that (±)-*trans*-caronic acid, m.p. 212°, was obtained by permanganate oxidation.

5). (±)-*cis*-Chrysanthemyl (±)-*cis*-Chrysanthemate (XIV). (±)-*cis*-Chrysanthemol chloride in 10 ml dry benzene was added into (±)-*cis*-chrysanthemol (1.5 g) and pyridine (1.5 ml) in 15 ml of benzene under cooling, the mixture was left standing two days at room temperature. The benzene solution was washed successively with 5% hydrochloric acid, water, 10% sodium carbonate solution and water. Benzene was recovered and distillation of the residue afforded the desired ester (2 g) boiling at 125°/0.078 mm, n_D^{23} 1.4835 (*Anal.* C, 78.01; H, 10.00. Calcd. for $C_{20}H_{32}O_2$: C, 78.89; H, 10.59%).

6). Oxidation of (±)-*cis*-Chrysanthemol with Potassium Permanganate. (±)-*cis*-Chrysanthemol (3 g) was suspended in 50 ml of water and powdered potassium permanganate (6 g) was added in small portions under vigorous stirring. After removal of manganese dioxide with suction, the filtrate was extracted with ether and the neutral matters were removed. The acid fraction was continuously extracted for 10 hrs. with ether after acidification of the alkaline solution, thus, ca. 3 g of acidic matter was obtained which partially crystallized, m.p. 95–100°. The crystals were divided into two portions: one was readily soluble and the other sparingly soluble in water. The former which melted at 100° and was proved to be oxalic acid by mixed melting point determination and the latter melted at 175°; the melting point of this portion was depressed when it was admixed with (±)-*cis*-caronic acid, terebic acid or succinic acid. Its structure is unambiguous. The non-crystalline portion was distilled and ca. 1 g of distillate at 120–130°/0.06 mm. was collected. Crystals melting at 136° gradually deposited in the distillate and the same crystals separated from the residue. The melting point was not depressed on admixture with authentic 2, 2-dimethyl succinic acid (XIII) (m.p. 138°).

7). (±)-Allylrethronyl-*cis*- and (±)-*trans*-Chrysanthemyl Ether (XX).

i). (±)-cis- *and* (±)-trans-*Chrysanthemol.* A mixture (15 g) of ethyl (±)-*trans*- and (±)-*cis*-chrysanthemate which was obtained by condensation of 2, 5-dimethyl-2, 4-hexadiene and ethyl diazoacetate[6]; after being redistilled once it was reduced with 1.7 g of lithium aluminum hydride in 50 ml of ether at 15° by an analogous method described above. The product redistilled at 91–2°/8 mm., n_D^{24} 1.4700, yield 10 g, 85% of the theoretical amount.

ii). *4-Bromo-allylrethrone* (*2-Allyl-4-bromo-3-methyl-2-cyclopentenone*). Phosphorus tribromide (9.6 g) and 5 ml of dry benzene were placed in a 50 ml four necked flask and 1.5 g of dry pyridine was added with stirring. To this a mixture of 15.2 g of allylrethrolone and 0.5 g of pyridine were gradually added at 5° and stirring was continued for 3 hrs after the addition was completed, then the mixture was left standing overnight. The reaction mixture was poured into ice water and extracted with benzene. The benzene extract was washed with water and distilled. 4-Bromo-allylrethrone distilled at 90–2°/0.05 mm., with partial decomposition, n_D^{17} 1.5432, yield 13 g, 60% of the theoretical amount.

iii). (±)-*Allylrethronyl* (±)-cis- *and* (±)-trans-*chrysanthemyl ether* (*XVII*). (±)-*cis*- and (±)-*trans*-chyrsanthemol mixture described above (8 g) and 2 g of sodamide in 30 ml of dry ether were stirred under reflux. After cooling 4-bromo-allylrethrone (10 g) was gradually added to the above mixture. Stirring was continued for 1 hr. then left standing overnight. After stirring and refluxing was performed for 1.5 hrs, and the mixture was washed with water and distilled. After distillation of 7 g of the low-boiling fraction the desired ether was obtained at 150–155°/0.05 mm., n_D^{19} 1.5347, yield 3.5 g (*Anal.* C, 80.0; H, 8.2. Calcd. for $C_{19}H_{28}O_2$: C, 79.1; H, 9.7%).

8). (±)-*cis*-Chrysanthemyl Bromide, (±)-*cis*-3-Isobutenyl-2, 2-dimethylcyclopropyl bromide (XV).

Five ml of benzene and phosphorus tribromide (5.6 ml) were placed in a 100 ml three necked flask then 1.7 ml (1.5 g) of pyridine was added under stirring and cooling at −50° (dry ice—acetone) then the mixture of (±)-*cis*-chrysanthemol (14 g), pyridine (0.5 g) and benzene (5 ml) was added dropwise at −20° under stirring. Stirring was continued further for an hour and left standing overnight at room temperature. The supernatant liquid was distilled and the fraction (16 g) at 63–66°/10 mm., n_D^{28} 1.4872 was collected.

9). Catalytic Reduction of (±)-*cis*-Chrysanthemic Acid in the Presence of Several Catalysts.

i). *Platinum oxide* (*Adam's catalyst*). (±)-*cis*-Chrysanthemic acid (17 g) in 100 ml of acetic acid was catalytically reduced in the presence of 200 mg of platinum oxide under ordinary atmospheric pressure. Hydrogen (2590 ml; 2540 ml is the theoretical amount) was consumed. The product was distilled and fraction boiling at 106–8°/0.3 mm was collected, of which 14.5 g gradually crystallized. Filtration and washing with petroleum ether afforded 12 g of the crystals melting at 54°. It does not decolorize cold potassium permanganate. *Anal.* C, 70.0; H, 10.3. $C_{10}H_{18}O_2$ requires: C, 70.6; H, 10.6%.

The crystals (8 g) were dissolved in 200 ml of 15% aqueous sodium hydroxide solution and oxidized with 8 g of potassium permanganate under stirring and warming in a water bath. Starting material (6.5 g) was recovered and from the water soluble acid fraction crystals, melting at 98° which were identical with authentic oxalic acid (m.p. 100°) by mixed melting point determination were obtained.

ii). *Palladous oxide.* In this case the results were identical with that of platinum oxide. The product melting at 54°, obtained, was identical with the acid, m.p. 54° mentioned above.

iii). *Raney-nickel catalyst.* a) (±)-*cis*-Chrysanthemic acid (10 g) was dissolved in 200 ml of 1% aqueous sodium hydroxide solution and was catalytically reduced by the 3 g of Raney-nickel under the initial pressure of 10 atm. The reduction was stopped after one equivalent of hydrogen was absorbed. The product was also the acid described above melting at 54°.

b). (±)-*cis*-Chrysanthemic acid (17 g) was dissolved in 400 ml of 1% aqueous sodium hydroxide solution and was shaken until two equivalent mole of hydrogen were absorbed, under the initial pressure of 12.5 atm., in the presence of 5 g of Raney-nickel. The acidic product was separated in the ordinary way and distilled, collected at 118–9°/8 mm. It was crystallized, filtered and washed with petroleum ether afforded crystals melting at 85° (*Anal.* C, 69.9; H, 10.3. Calcd. for $C_{10}H_{20}O_2$: C, 69.8; H, 11.6%). *p*-Phenylphenacyl ester was prepared, mp. 69–71° (*Anal.* C, 78.9 H, 7.7. Calcd. for $C_{24}H_{30}O_3$: C, 78.8; H, 8.2%). This acid decolorized potassium permanganate in the cold and it was assumed to be VII which has not already a cyclopropane ring.

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[6] I.G.M. Cambell and S.H. Harper, *J. Chem. Soc.* **1945**, 283.

The crystals (10 g) mentioned above were dissolved in 200 ml of 20% aqueous sodium hydroxide solution and oxidized by gradually adding 10 g of powdered potassium permanganate on the steam bath under stirring. The acidic product was separated in the ordinary way and distilled and the fraction boiling at 113–155°/8 mm. was collected, 8 g. This was dissolved in ether and was washed thoroughly with saturated sodium bicarbonate solution. After evaporation of the solvent, the residue was distilled and a fraction boiling at 119–121°/8 mm. was obtained, 6.8 g. It crystallized on standing, melted at 55° when washed with petroleum ether. (*Anal.* C, 71.42; H, 9.68. Calcd. for $C_{10}H_{18}O_2$: C, 70.7; H, 10.6%). It did not decolorize potassium permanganate in the cold. *p*-Phenylphenacyl ester was prepared, m.p. 80°. Analytical data showed that dehydration occurred in the process (*Anal.* C, 80.57; H, 6.83. Calcd. for $C_{24}H_{30}O_4$, C, 75.4; H, 7.8% and Calcd. for $C_{24}H_{28}O_3$: C, 79.1; H, 7.1%). Mixed melting point determination showed that the lactone was not identical with Harper's (±)-dihydrochrysanthemolactone and (±)-pyrocin. This substance is probably dihydropyrocin (XI).

The sodium bicarbonate-soluble fraction described above was acidified and the acid was extracted and distilled. The fraction boiling at 145–155°/8 mm. crystallized. Washing with petroleum ether—ethyl acetate afforded crystals melting at 144–5° (*Anal.* C, 54.33; H, 7.77. Calcd. for $C_7H_{10}O_4$: C, 53.2; H, 6.3%). This acid may probably the iso-propenylsuccinic acid, (m.p. 146°) but this was not confirmed.

10). (±)-*trans*-δ-chloro-dihydrochrysanthemic acid (VIII). (±)-*trans*-δ-hydroxydihydrochrysanthemic acid (IV) (42 g) and acetyl chloride 44 g (40 ml) were mixed and warmed at 40°. Crystals disappeared into the solution. After cooling, the mixture was poured into 400 ml of ice water and extracted with petroleum ether (500 ml). The extract was washed and filtered. Upon evaporation of the solvent it crystallized. Recrystallization from petroleum ether afforded 25 g of crystals melting at 91–2° (C, 59.89; H, 7.81; Cl, 16.14. Calcd. for $C_{10}H_{17}O_2Cl$: C, 58.6; H: 8.3, Cl: 17.4%). Mother liquor was distilled and fractions a) ~115°/0.4 mm, 4.3 g, b) 115–122°/0.4 mm, 2.7 g, c) 122–124°/0.4 mm, 11.2 g were obtained. Fraction c) crystallized, and was washed with petroleum ether, m.p. 91–2°, 5 g. This is identical with the crystals described above. The total yield was 30 g.

11). (±)-*trans*-Chrysanthemic acid. (±)-*trans*-δ-chlorodihydrochrysanthemic acid (20 g) and 40 g of diethylaniline were mixed and heated at 180–220° for 2 hrs., then poured into the mixture of 50 ml of hydrochloric acid containing 200 g of ice and extracted with ether. The ether extract after being washed once with 5% hydrochloric acid and thrice with water, was distilled. The distillate (14.4 g, 88%) boiling 110°/0.1 mm became crystal mass m.p. 48°. Recrystallization from ethyl acetate afforded pure (±)-*trans*-chrysanthemic acid melting at 54° which was confirmed by mixed melting point determination. Collidine was also used successfully, but dehydrochlorination with pyridine was failed.

Most of these experiments were carried out at the Laboratory of Sumitomo Chemical Co., Ltd., Osaka Works, in 1953. The authors with to express their thanks to the director of Osaka Works for his generous allowance of this publication. They also wish to express their thanks to Prof. Yusuke Sumiki for his interest in this work.

Short Communications

On Reduction of Agroclavine and Elymoclavine with Sodium and *n*-Butanol

Sir:

It is believed that agroclavine (I) gives two isomeric dihydro-derivatives by reduction of the double bond between carbon atoms 8 and 9 as in the case of elymoclavine[1]. However, as a matter of fact, the catalytic hydrogenation of this substance[2,3] merely yields one dihydro-derivative, which is identical with festuclavine (alkaloid "Y") (II).

Recently, we have studied the reduction of agroclavine with sodium and *n*-butanol in the hope that the supposed derivative would result. The reduction gave a reaction mixture from which a number of substances, viz., pyroclavine (III), costaclavine (IV) and a basic substance (V), not to speak of festuclavine (II), were isolated. The basic substance was crystallized from ethyl acetate, methanol or ethanol in prisms; m.p. 265–7° (uncorr. decomp.), $[\alpha]_{\mathrm{D}}^{30}=+67°$, $[\alpha]_{5461}^{30}=+99°$ (c. 0.2 in pyridine), $[\alpha]_{\mathrm{D}}^{30}=+112°$, $[\alpha]_{5461}^{30}=+149°$ (c. 0.2 in $CHCl_3$). *Anal.* Found: C, 80.90; H, 7.73; N, 11.70; N–CH_3, 12.23. Calcd. for $C_{16}H_{18}N_2$: C, 80.62; H, 7.62; N, 11.76; N–CH_3, 12.19%. It gave the color reactions of agroclavine and showed the UV.-spectrum typical of lysergic acid. It was insoluble in petroleum ether, sparingly soluble in ether, benzene, ethyl acetate, acetone, methanol and ethanol, but moderately soluble in chloroform and pyridine. Its solutions in organic solvents exhibited a marked fluorescence. It dissolved slightly in water, and readily in dilute acids. This basic substance exclusively yielded festuclavine (II) on catalytic hydrogenation, whereas, it afforded the same substance together with a considerable amount of costaclavine (IV) on the reduction with sodium and *n*-butanol. Moreover, the basic substance itself was obtained in excellent yield by treatment of agroclavine with hot sodium butylate. These facts indicate that the substance corresponds to 6, 8-dimethyl-ergolene-(9) (V). We have therefore adopted the designation lysergine for this product.

It is naturally presumed that elymoclavine (VI), on reduction with sodium and alcohols, gives a certain lysergol as a product corresponding to lysergine, besides different dihydrolysergols. However, when elymoclavine was reduced with sodium and *n*-butanol by a similar procedure as in the case of agroclavine, an unexpected result was obtained. That is, the reduction gave agroclavine (I), festuclavine (II), pyroclavine (III), costaclavine (IV) and lysergine (V), as well as α- and β-dihydrolysergols (VII and VIII, respectively). In the meantime, it was ascertained that both α- and β-dihydrolysergols once occurred, could never suffer reduction of their alcohol groups to the methyls during the procedure. From this fact and from the result obtained in the reduction of agroclavine, it is evident that in the above reduction the following reactions have proceeded, thus giving the conclusion that the stereochemical constitutions of agroclavine (I), festuclavine (II), pyroclavine (III), costaclavine (IV), lysergine (V) and elymoclavine (VI) must be those as shown here.

At any rate, the present results give support to the previous presumption[3,4] that agroclavine must be produced by ergot fungus

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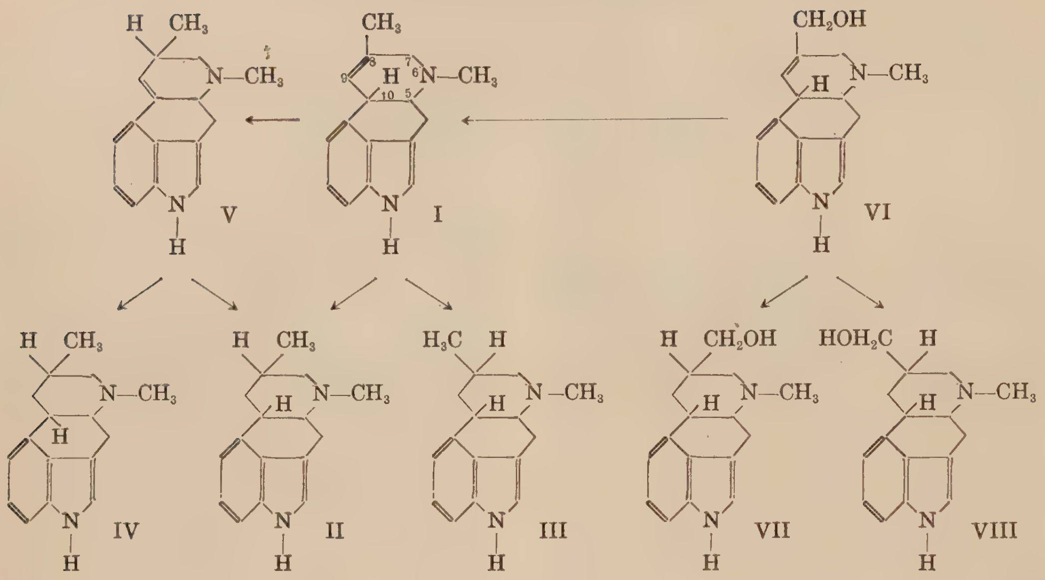
[4] M. Abe, T. Yamano, Y. Kozu and M. Kusumoto, Report at the Annual Meeting of Agr. Chem. Soc. Japan held at the University of Tokyo (April 5, 1952).



through elymoclavine, as well as our view[5] that like festuclavine, both pyroclavine and costaclavine are produced through agroclavine by the fungus.

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Isolation and Identification of Taraxasterol from *Balanophora japonica* Makino*

Sir:

Balanophora japonica Makino is an epiphytic plant belonging to *Balanophoraceae*, and it is reported to be indigenous to the southern part of Japan, and in Loochoo. To date, taraxasterol has been reported to be present in several plants belonging to *Compositae*[1)~6)] and to *Euphorbiaceae*[7)]. It is considered of interest, here, to note that taraxasterol has now been confirmed to be present in *Balanophora japonica* Makino.

The Procedure of Fractionation: The rhizomes and flowers (1.54 Kg.) of the above plant were cut into chops, air-dried, and thoroughly dried at 60° for 10 hrs. Yield: 435.0 g. (28.24%). The unsaponifiable fraction of the ethereal extract (49.0 g.: 11.26% of the dried matter) was acetylated so as to yield a mixture of taraxasteryl acetate and β-amyrin acetate, which was fractionally recrystallized from alcohol and then from ethyl acetate. From the fraction sparingly soluble in alcohol, taraxasteryl acetate[8)] was isolated in pure form as glistening scaly plates (3.7 g.) from ethyl acetate, $C_{32}H_{52}O_2$, $[\alpha]_D^{12}+100.0°$ (c, 0.634, $CHCl_3$), m.p. 248–249°. *Anal.* Found: C, 81.55; H, 10.10. Calcd. for $C_{32}H_{52}O_2$: C, 81.99; H, 11.18. Deacetylation of the acetate afforded taraxasterol, $C_{30}H_{50}O$, $[\alpha]_D^{12}+93.5°$ (c, 0.852, $CHCl_3$), m.p. 220–221°, colorless long fine needles from alcohol. *Anal.* Found: C, 84.04; H, 11.82. Calcd. for $C_{30}H_{50}O$: C, 84.44; H, 11.81. Taraxasteryl monobenzoate[8)], $C_{37}H_{54}O_2$, $[\alpha]_D^{12}+106.5°$ (c, 0.595, $CHCl_3$), m.p. 242°, needles from acetone-benzene. *Anal.* Found: C, 83.11; H, 10.28. Calcd. for $C_{37}H_{54}O_2$: C, 83.72; H, 10.25. The compound was further identified as taraxasterol by isomerizing it with 7% sulfuric acid into ψ-taraxasterol[9)] (heterolupeol), $C_{30}H_{50}O$, $[\alpha]_D^{12}+48.5°$ (c, 0.652, $CHCl_3$), m.p. 208–209°, long fine needles from alcohol. *Anal.* Found: C, 83.47; H, 11.73. Calcd. for $C_{30}H_{50}O$: C, 84.44; H, 11.81. ψ-Taraxasteryl monoacetate, $C_{32}H_{52}O_2$, $[\alpha]_D^{12}+56.2°$ (c, 0.752, $CHCl_3$), m.p. 238–240°, leaflets from ethyl acetate-alcohol. *Anal.* Found: C, 81.81; H, 11.12. Calcd. for $C_{32}H_{52}O_2$: C, 81.99; H, 11.18.

β-Amyrin, m.p. 196°, $[\alpha]_D^{13}+86.2°$ (c, 0.583, $CHCl_3$), was isolated from the fraction readily soluble in alcohol, and identified as its monoacetate, $C_{32}H_{52}O_2$, $[\alpha]_D^{13}+83.3°$ (c, 0.665, $CHCl_3$), m.p. 239°, and monobenzoate, m.p. 232°. Palmitic acid, m.p. 62°, was isolated from the fatty acid fraction, and identified as its acid amide, m.p. 106°.

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On the Migration Distance of Amino-acids in Paperelectrophoresis

Sir:

We conducted an experiment on the migration distance of amino acids under various conditions of paperelectrophoresis. Filter-paper (Tōyō, No. 50, 2×30 cm, 2×40 cm) treated with N-HCl and N-NaOH were put in a phosphate buffer solution. The above apparatus was placed in a thermostat and kept at 26.5～27.0°C under saturated vapour pressure. After more than thirty minutes since the buffer soaked through the filterpaper, the current was turned on for about twenty minutes, and then the amino acid solutions were dropped on the original points. In electrophoresis the voltage was kept constant and the current was measured at an interval of five minutes, their arithmetical mean being assumed as the average current.

Glycine migrated to the cathodic side in pH 7.38, and the migration distance increased in inverse proportion to the average current, its mobility gradually decreasing with the lapse of time. Each amino acid had a static point on filterpaper under each condition and migrated towards them. Increase of current shifted the static points of amino acids toward the anodic side. After electrophoresis, the amino acids held the same relative distance from each other without reference to their dropped points. The migration distance of other amino acids from glycine was about twice when the voltage was doubled. From our experiment, it was found that the length of filterpaper had effect on the migration distance.

If we take the following ratio as MG of amino acids,

$$MG = \frac{\text{migration distance of other amino acids from glycine}}{\text{migration distance of aspartic acid from glycine}},$$

then, MG is free from current, voltage, the dropped point, length of filterpaper and time of electrophoresis, but not from pH of the surrounding buffer solution. The MG of amino acids in this definition are shown in Table. I.

TABLE I

MG OF AMINO ACIDS IN VARIOUS pH OF PHOSPHATE BUFFER

amino acid pH	aspartic acid	glutamic acid	lysine arginine	histidine	neutral amino acids
8.30	+1.00	+0.88	−0.52	−0.06	0
7.38	+1.00	+0.80	−0.60	−0.22	0
6.47	+1.00	+0.74	−0.85	−0.50	0
5.59	+1.00	+0.66	−0.99	−0.80	0
4.53	+1.00	+0.53	−1.18	−1.08	0

In our experiment it was impossible to avoid the effect of Joule's heat and the change of pH on parts of filterpaper.

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Studies on Microbial Biosynthesis of Pantothenic Acid

Sir:

Regarding pantothenic acid synthesis from pantoic acid and β-alanine Maas and Novelli[1] have shown that the overall reaction is: pantoate + β-alanine + ATP → pantothenate + AMP + PP, and the following sequence has been presented by Mass[2] as the mechanism of this reaction: 1) Enzyme (E) + ATP ⇋ E-PP + AMP, 2) E-PP + pantoate ⇋ E-pantoate + PP, 3) E-pantoate + β-alanine ⇋ pantothenate + E.

The data indicated in this paper suggest that activation of the carboxyl group of pantoate occurs at the expense of the PP bond of ATP and pantothenate synthesis takes place anaerobically in the presence of ATP. In the hope of trapping the activated carboxyl group of the intermediary compound containing the enzyme, hydroxamate formation was employed (Table I). 0.2 ml of the extract, prepared from acetone-dried cells of *Escherichia coli* by the method of Maas,[3] was incubated for 1 hour at 30° in a final volume of 1.0 ml with 10μM K-pantoate, 10μM β-alanine, 5μM ATP (Ksalt), 100μM KCl, 20μM $MgCl_2$, and 100μM tris (hydroxymethyl) aminomethane buffer pH 8.6. Analyses:

TABLE I

APPEARANCE OF HYDROXAMATE IN PANTOTHENATE SYNTHESIS

(in μM/ml; incubation, 1 hr.)

System	Hydroxamate found	Acid-labile phosphate* lost	Inorganic phosphorus** found
Complete	0.32	2.17	2.15
Without β-alanine	0.58	1.07	1.08
〃 K-pantoate	0	1.10	1.08
〃 ATP	0.02	0	0
〃 β-alanine & ATP	0	0	0
Without β-alanine, with 800μM NH_2OH	0.59	1.04	1.05

* as ATP, ** as PP

(1) Pantothenate was determined according to the method of Bergmann[4] (at 505 mμ).

(2) Hydroxamate of activated pantoate was measured by the following procedure: 1.0 ml of the enzymatic reaction mixture was incubated for 10 hours at −5° with 2.0 ml of 1N hydroxylamine reagent pH 9.4 containing Mn^{++} (added with 0.15% $MnCl_2$ and filtered), after rapid cooling by the freezing mixture. At the end of the incubation 0.5 ml of 3N HCl and then 0.5 ml of 40% trichloroacetic acid was added, centrifuged, adjusted to around pH 1.2 with 0.5 ml of 2.0-2.5N NaOH. The color developed by the addition of 0.5 ml of 1.48M ferric chloride in 0.2M HCl-KCl buffer pH 1.2 was measured at 505 mμ and compared to a zero-time control using hydroxamate of (−)-pantolactone as a relative standard. Under these conditions, the color increase of a zero-time control was negligible, although the enzymatic reaction mixture was simply cooled at −5°.

(3) At the end of incubation the reaction mixture was treated with trichloroacetic acid at 0° and inorganic phosphorus was determined according to the method of King[5] on the precipitate by $CaCl_2$ reagent which had been hydrolysed for 15 minutes in 1N HCl.

(4) Acid-labile organic phosphate was similary determined on the supernatant hydrolysed for 15 minutes in 1N HCl which was separated from the above precipitate.

A significant quantity of hydroxamate was found when pantoate was incubated with the enzyme in the presence of ATP. But

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Abbreviations: ATP-adenosinetriphosphate; AMP-adenosine-monophosphate; PP-inorganic pyrophosphate.

[1] W.K. Maas and G.D. Novelli: *Arch. Biochem. and Biophys.*, **43**, 236 (1953); [2] W.K. Mass: *Federation Proc.*, **13**, 256 (1954); [3] W.K. Maas: *J. Biol. Chem.*, **198**, 23 (1952); [4] F. Bergmann: *Anal. Chem.*, **24**, 1367 (1952); [5] E. J. King: *Biochem. J.*, **26**, 292 (1932),

when the reaction mixture was heated at 100° or treated with the strongly alkaline hydroxylamine reagent, the color of hydroxamate was greatly decrease. This fact observed under the protein-denaturating conditions may suggest that the intermediate containing activated pantoate is labile and the activated carboxyl group is directly attached to the enzyme. Formation of pantothenate and activation of pantoyl-carboxyl group increase under the anaerobical conditions (Table II).

TABLE II

ANAEROBIC SYNTHESIS OF PANTOTHENATE

(in μM/ml; incubation, 1 hr.)

System	Gas phase	Pantothenate found
Complete	Air	0.75
〃	N_2	1.16
〃	Vacuum	1.14
Without ATP	Air	0

Inhibition of pantoyl-carboxyl group activation by -SH reagents and reversal by reducing reagents are indicative of the evidence for involvement of -SH enzyme in this reaction (Table III). These findings permit the possibility that activated pantoate may be in the form of a reactive complex with the -SH groups of the enzyme.

TABLE III

INHIBITION OF PANTOTHENATE SYNTHESIS AND PANTOATE ACTIVATION BY -SH REAGENTS; REVERSAL BY REDUCING REAGENTS

(in μM/ml; incubation, 1 hr.)

System	Pantothenate found	Activated pantoate found
Complete	0.71	—
With 12μeq. H_2O_2	0.13	—
〃 +17μM thioglycolate	0.49	—
With 0.5μM *p*-chloromercuribenzoate	0.24	—
〃 +17μM thioglycolate	0.68	—
Without β-alanine	0	0.54
〃 , with 0.5μM *p*-chloromercuribenzoate	—	0.08
〃 , 〃 +17μM thioglycolate	—	0.34
〃 , 〃 +17μM glutathione	—	0.50

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